## Discovery of the wound-healing capacity of salivary histatins

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2010

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# Chapter 6

## Receptor-mediated uptake of histatin is independent of cell activation

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Submitted for publication

## Abstract

Histatins (Hsts) are a family of multifunctional peptides present in saliva of human and some non-human primates. Recently, we found that certain members of the Hst family (Hst1 and Hst2) potently enhance epithelial cell migration *in vitro* and re-epithelialization *ex vivo*, and thus are likely to be involved in the protection of the soft tissue of the oral cavity. The interaction of Hsts with their target cells display characteristics resembling those of regular growth factors, which upon binding are taken up *via* endocytosis. Hsts are likewise internalized through a stereo-specific, energy-dependent interaction with a putative receptor. In this study, we examined whether there is a correlation between the cell-stimulating activity of Hst species, and the rate by which they are internalized by their target cell. We found that the specific activity of Hsts decreased in the order Hst2>Hst1>>Hst3~Hst5, whereas their rate of uptake by the cells was virtually identical. Hst2 effectively blocked uptake of Hst5, indicating competition for the same receptor. These data suggest that activation of the Hst receptor is not a prerequisite for uptake.

## Introduction

The histatin (Hst) family of peptides is present in the saliva of certain primate species including that of humans (1). The human genome contains two Hst genes: *HTN1* giving rise to Hst1 and *HTN3* giving rise to Hst3. All other Hsts arise due to proteolytic degradation of these two parent gene-products. Hst1, Hst2, Hst3, and Hst5 are responsible for the bulk of Hsts in human saliva. Their function seems to be versatile but generally relates to the protection of the hard and soft tissues in the mouth. Protease-inhibition, metal-binding, tannin-binding, antimicrobial activity, and recently also cell-migration activities, all of which have been described for Hsts (2-6).

We have previously found that Hst-induced cell migration possesses the characteristics of a ligand-receptor mediated process. It involves binding to a stereo-specific cellular receptor, followed by energy-dependent endocytosis of the cell (7). In addition, the activation of cell migration by Hst is pertussis-toxin sensitive, which implies the involvement of a G-protein coupled receptor (GPCR) (3). Nowadays, over 900 GPCRs have been identified, several of which have no known ligand (8). In general, receptor-mediated processes are regulated tightly at several levels to avoid continuous signalling. One of these levels proceeds through endocytosis of the receptor-ligand complex.

The aim of this study was to examine if there is a correlation between the cell-stimulating activity of the major salivary Hsts (Hst1,2,3,5) and the rate by which they are endocytosed. We found that the cell-migration activity, as measured by an *in-vitro* wound-closure model, of Hsts decreased in the order Hst1~Hst2>>Hst3~Hst5. The uptake rate of the different Hsts however, was virtually identical. We also found that Hst2 effectively obstructed the uptake of Hst5, an indication for the competition of the same receptor. Our data suggest that activation of the putative Hst receptor is not a prerequisite for its cellular uptake.

## Material and Methods

### Cell-Line Culture

Human buccal-epithelial cell line HO-1-N-1 was provided by the Japanese Collection of Research Bioresources (Osaka, Japan). Cells were cultured in DMEM-F12 medium (Invitrogen, Carlsbad, CA, USA), fully supplemented (10 % fetal calf serum (HyClone, South Logan, UT, USA), 2 % antibiotic-antimycotic solution (100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B, Sigma-Aldrich, St. Louis, MO, USA)), at 37 °C, 95 % humidity and 5 % $CO_2$. Cells were maintained until confluence, detached with 0.25 % trypsin-EDTA (Invitrogen), counted with hemacytometer, and seeded into new flasks or multiwell plates at the required cell densities.

### Peptide Synthesis

Peptides were synthesized by solid-phase peptide synthesis using Fmoc chemistry with a MilliGen 9050 peptide synthesizer (Milligen-Biosearch, Bedford, MA, USA). Purification by RP-HPLC and confirmation of authenticity by MS were conducted as described previously (9). Amino-acid sequence, molecular weight, net charge, and amphipaticity of the peptides used in this study are depicted in Table 1.

**Table 1** Amino acid sequence, molecular weight (MW), charge, and amphipaticity of Hsts.

| Peptide | Amino acid sequence | MW | Charge[a] | <µH>[b] |
|---|---|---|---|---|
| Hst1 | D**S**HEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN | 4848 | +1 | 0.156 |
| Hst2 | RKFHEKHHSHREFPFYGDYGSNYLYDN | 3445 | 0 | 0.137 |
| D-Hst2 | rkfhekhhshrefpfygdygsnylydn | 3445 | 0 | 0.137 |
| Hst3 | DSHAKRHHGYKRKFHEKHHSHR.....G.YRSNYLYDN | 4062 | +5 | 0.156 |
| Hst5 | DSHAKRHHGYKRKFHEKHHSHR.....G.Y | 3037 | +5 | 0.210 |

**S** = phosphoserine.

[a] Net charge at pH=7.

[b] <µH> amphipaticity, calculated as mean hydrophobic moment at 100°, in an α-helical conformation. (20)

### *In vitro* Wound-Closure Assay

Epithelial wound-closure experiments were performed as described previously (10). Cells were grown in 48-well plates until confluence, and serum deprived for 24 h in prescribed medium without additives. In each well a scratch was made using a sterile tip and cellular debris was removed by washing. The width of the scratch was determined microscopically, by making one representative micrograph

per well, immediately after creation and 20 h later. Relative closure of the scratch was calculated as $(X_o - X_{yy})/(C_o - C_{yy})$, in which: $X_o$ = width of the scratch at time = 0; $X_{yy}$ = width of the scratch after yy time exposure to a condition; $C_o$ = width of the scratch at time = 0; $C_{yy}$ = width of the scratch after yy time exposure to the control.

**Cytotoxicity**

HO-1-N-1 cells were seeded in 96-wells plates at $2x10^4$ cells per well in DMEM/F12 fully supplemented. Cells were serum-starved (without serum or antibiotics) for 6 h, after which the medium was refreshed and the different conditions (Hst2 100 μM, Hst5 100 μM, control is medium alone, without Hsts) were applied. After 20 h of incubation propidium iodide (PI) (final concentration 15 nM) was added to the medium. As a positive control, 20 min before PI addition, Triton X100 (final concentration 3 %) was added to a set of control wells. Fluorescence was measured at excitation and emission wavelengths of 544 and 620 nm, respectively, in a Fluostar Galaxy microplate fluorimeter (BMG Labtechnologies, Offenburg, Germany).

**RP-HPLC measurements of depletion of histatins from medium**

$2x10^5$ cells were seeded in a 48-well plate and grown for 48 h in supplemented prescribed medium. Then the cells were washed twice with keratinocyte SFM (Invitrogen) and SFM medium containing peptide, peptide combinations, or peptide and protease-inhibitor cocktail (complete mini, Roche, Indianapolis, IN, USA) / PMSF (1 mM) /isopropanol (PMSF control) / EDTA (1 mM) /EGTA (1 mM) were applied. Peptide concentrations are as indicated in the figures. Immediately after application and after a certain incubation time aliquots were taken, possible debris was removed by centifugation and the concentration of Hsts was determined on an analytical C-18 column (Vydac, Grace, Deerfield IL, USA) on a Jasco HPLC system with a gradient of 5-65 % acetonitrile in 25 minutes at 1 ml/min. Absorbance was measured at 214 nm. Percentage removed peptide from medium was calculated as $(Area_{t=0}) - (Area_{t=x})/(Area_{t=0})*100$. Wells without cells were commonly used as a control in the experiments and never a 'removal from medium' in these wells was observed (data not shown).

## Results

### Cell-stimulating activities of Hst1, Hst2, D-Hst2, Hst3, and Hst5

We have previously shown that several members of the salivary Hst family stimulate cell migration (7) and are endocytosed in an energy-dependent and stereo-specific process (7). Here, we examined whether cellular activation by Hsts and their endocytosis are obligatory linked processes. First, we characterized the concentration-dependent cell-activating potencies of Hst1, Hst2, Hst3, and Hst5. The different peptides were tested in an artificial wound model using a concentration range between 0.1-100 μM (Fig. 1). Hst1 and Hst2 stimulated wound closure maximally at 10 μM. In contrast, Hst3 and Hst5 exerted only stimulatory effect at the highest concentration tested, 100 μM. The level of wound closure by Hst3 and Hst5, however, did not reach the maximal level as observed with Hst1 and Hst2. On the other hand, D-Hst2 did not induce wound closure at any concentration, indicative for the stereo-specificity of the activation by Hst1 and Hst2.

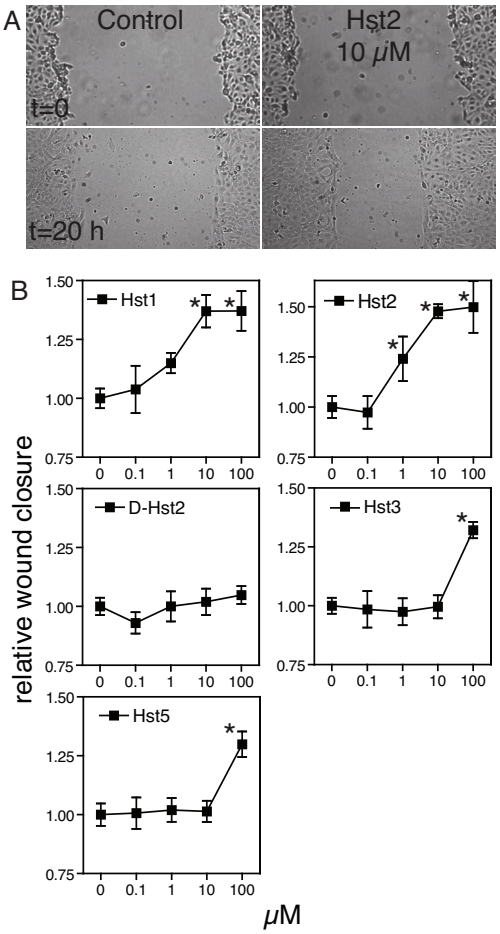


**Figure 1** Dose response curves of *in vitro* wound closure of Hst1, Hst2, D-Hst2, Hst3, and Hst5.

**A** Representative images of a typical *in vitro* wound closure experiment. Wound scratch at t=0 (upper panel) and t=20 (lower panel) with and without Hst2 (10 μM) in a confluent layer of HO-1-N-1 epithelial cells. **B** Relative wound closure is calculated as described in the Material and Methods section. Dose response curves (0-100 μM) show differences in activity between Hsts. Dot represents mean±SD, n=10, * $p < 0.05$ tested with one-way analysis of variance (ANOVA) with additional least significant difference (LSD) test compared to untreated control.

Since Hsts, particularly Hst5, are potentially membrane-active (11), it could be possible that they exert adverse effects on epithelial cells. Therefore, we tested membrane integrity and subsequent cytotoxicity of the epithelial cells using propidium iodide (PI) in the presence of 100 μM Hst2 or Hst5. As a positive control the detergent Triton X-100 was used. PI only enters membrane-compromised cells, after which the fluorescence of this probe is enhanced 20- to 30-fold due to its binding to nucleic acids. We exposed epithelial cells to 100 μM Hst2 or Hst5 for 24 h, and measured cell-associated PI-fluorescence. Neither Hst2 nor Hst5 had compromising effects on the membrane barrier function (Fig. 2).

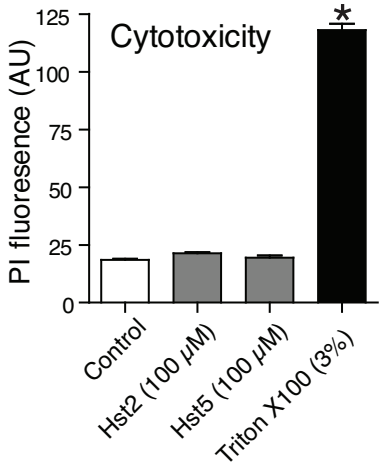


**Figure 2** Hsts are not cytotoxic due to membrane permeabilization.
PI fluorescence was used to measure cytotoxicity, assuming that membrane permeabilization is followed by cell death. No cytotoxic effects could be seen, even for the most positively charged Hst5. Triton X100 was used as positive control. Bar represents mean±SD, n=8, * $p < 0.05$ tested with one-way ANOVA with additional LSD test.

## Hst uptake by epithelial cells is not correlated to Hst-activating properties

To study endocytosis rates we designed an HPLC-based assay in which we measured the reduction of peptide from the medium as an indication for uptake by the cells. In Figure 3A an example is shown of the reduction of Hst2 in the medium (10 μM) after 20 h. We compared the uptake of the Hsts that are most abundantly present in saliva. No differences between peptides could be detected, except for the stereo-specificity of the process (Fig. 3B), D-Hst2 is not taken up at all compared to the L-Hsts. To examine if the observed decrease in the medium was caused by proteolysis, we incubated Hst2 in the presence of a protease inhibitor cocktail (Fig. 3C). The uptake was almost completely abolished. However, when we examined the ingredients of the protease inhibitor cocktail separately we found that it is not the protease inhibitors (PMSF) that reduce uptake, but rather that it is EDTA that abrogate uptake (Fig. 3C). The next obvious check was to see whether the uptake was also EGTA-sensitive, which turned out not to be the case.

Then we tested Hst2 depletion from medium in time, and we observed a biphasic uptake of Hst2 (Fig. 3D). This may correspond to receptor-mediated endocytosis, after the initial depletion of Hst2 the uptake of Hst2 is dependent on the recycling of the putative receptor back to the membrane.

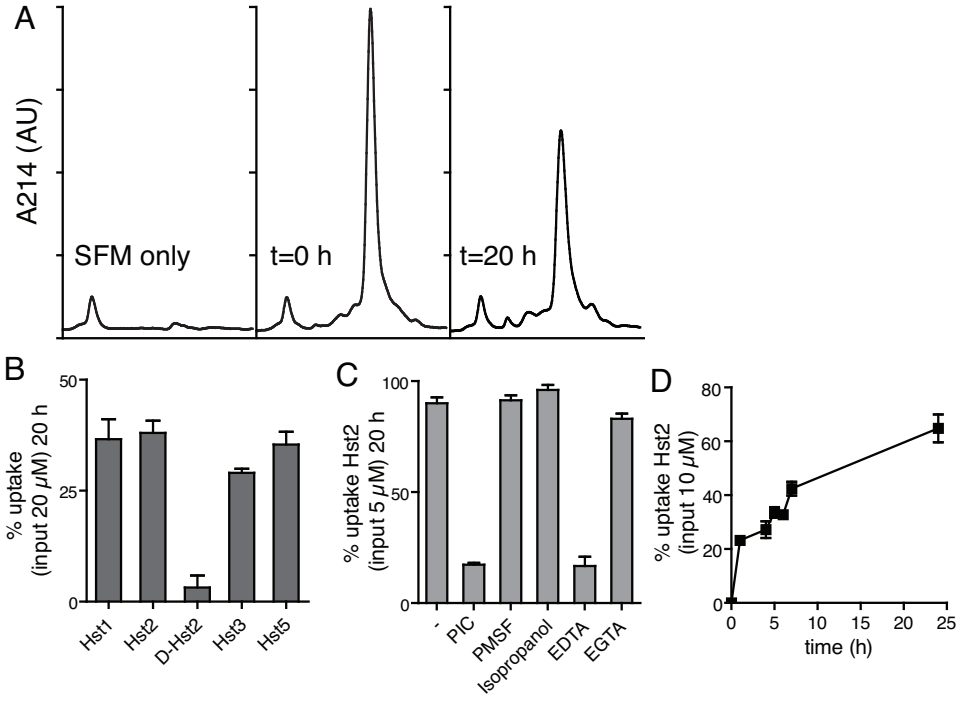


**Figure 3** Stereospecific and calcium independent endocytosis of Hsts as measured with RP-HPLC.

**A** Part of RP-HPLC profiles of SFM only, SFM + Hst2 (10 μM) immediately after addition (t=0) and at t=20 h. Area of Hst peak at t=0 is calculated and set to 100 %, reduction of peak is calculated as % uptake (see also material and methods). Control empty wells did not show peak area reduction (data not shown) **B** Uptake by different Hsts. Apart from the stereospecific uptake (D-Hst2 is not taken up) no differences between Hsts is observed. **C** Hst2 (input 5 μM) uptake under different conditions. PIC (Protease inhibitor cocktail) and EDTA inhibit uptake whereas PMSF, isopropanol, and EGTA does not. (B & C) Bar represents mean±SD, n=3. **D** Hst2 percentage removed from medium after different time points, with 10 μM as input. Biphasic character of line is typical for receptor-mediated endocytosis of ligands. Dot represents mean±SD, n=3.

**Hst2 has a higher binding affinity compared to Hst5**

To explore if Hsts compete for the same putative receptor, we studied the mutual inhibitory effects on their uptake by the cell. We chose to compare Hst2 and Hst5 as they are the most diverged in activation of epithelial cells. Hst2 uptake was slightly but significantly reduced upon a co-incubation with a 10-fold excess of Hst5 (Fig. 4A). In contrast, Hst5 uptake was drastically decreased in the presence of a 10-fold excess of Hst2 (Fig. 4B). These results points to the mediation of the uptake of Hst2 and Hst5 by a common receptor. In addition, Hst2 has a higher affinity for this receptor than Hst5, which corresponds to their cell-activating properties.

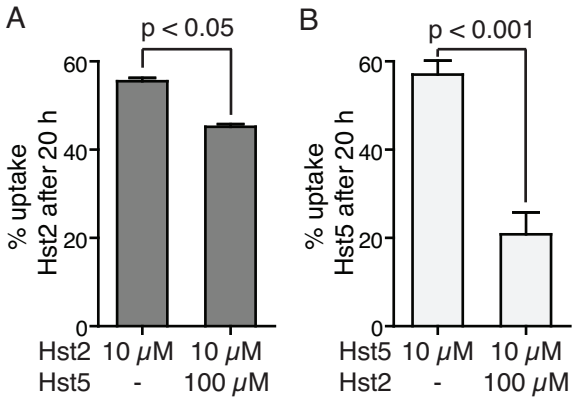


**Figure 4** Different uptake affinities between Hst2 and Hst5 as measured with competition for uptake.

**A** Hst2 (10 μM) uptake is slightly reduced in the presence of excess Hst5 (100 μM). **B** Hst5 (10 μM) uptake is severely reduced in the presence of excess Hst2 (100μM). Bars represent mean±SD, n=6, p values are in the figure, student t-test was used.

## Discussion

In this study we have compared the wound-closure activity and endocytosis rate of the Hsts that are most prominently present in saliva. We found that wound-closure activity of the different Hsts differ greatly, whereas their endocytosis rates were equal. However, in a competition assay, we found that Hst2 has a higher binding affinity than Hst5, and this does correspond with the higher activity of Hst2 compared to Hst5. This competition assay also indicated that Hst5 and Hst2 probably act *via* the same receptor. Nevertheless, both the uptake as the activity requires a stereo-specific interaction.

Our finding that non-activating concentration of Hst5 (10 μM, Figs. 1 & 4) is endocytosed equally well as the potent Hst2 (also 10 μM, Figs. 1 & 4) suggests that the receptor-mediated uptake is independent from activation. Others have found that the speed of a ligand to promote endocytosis does not linearly correlate with ligand activity (12, 13). In addition, an antagonist can also induce endocytosis, thus without activation whatsoever (14-16). Also, a model for class-B GPCRs exists in which a secondary binding is necessary for receptor activation, thus after an initial primary binding that alone does not suffice for activation (17). Our findings fit well in the emerging picture of endocytosis of GPCR-ligand complexes, in which activation and endocytosis are not necessarily linked.

We previously found cell-activating properties for Hst3, but not for Hst5 (7). Another study, that only tested Hst3 and Hst5, found some cell-activation for Hst5 but more for Hst3 (18). This study together with our initial discovery (7) confirms with Imamura *et al* (18) that Hst5 has activity but that Hst3 is more active. The biological relevance of this is matter of debate, since *HTN2* gene products (Hst3 and Hst5) are quite vulnerable for proteolysis in saliva compared to *HTN1* gene products (Hst1 and Hst2) (19).

In conclusion, our findings are additional proof of the existence of a cellular receptor for Hsts. The differences in affinity are potentially interesting to study in more detail. The ease by which peptides can be altered or modified together with uptake-affinity competition assays may form an excellent basis for the search for the specific residues responsible for binding and activation. Furthermore, Hsts remain fascinating peptides with high potential for drug development for wound healing therapeutics with no demonstrable adverse effects on host cells.

## Acknowledgements

Enno C.I. Veerman gratefully acknowledges the support from the Skeletal Tissue Engineering Group Amsterdam (STEGA).

## References


1. Padovan, L., Segat, L., Pontillo, A., Antcheva, N., Tossi, A., and Crovella, S. (2010) Histatins In Non-Human Primates: Gene Variations and Functional Effects. *Protein Pept Lett*
2. Nishikata, M., Kanehira, T., Oh, H., Tani, H., Tazaki, M., and Kuboki, Y. (1991) Salivary histatin as an inhibitor of a protease produced by the oral bacterium Bacteroides gingivalis. *Biochem Biophys Res Commun* 174, 625-630
3. Oudhoff, M. J., Kroeze, K. L., Nazmi, K., van den Keijbus, P. A., van 't Hof, W., Fernandez-Borja, M., Hordijk, P. L., Gibbs, S., Bolscher, J. G., and Veerman, E. C. (2009) Structure-activity analysis of histatin, a potent wound healing peptide from human saliva: cyclization of histatin potentiates molar activity 1,000-fold. *Faseb J* 23, 3928-3935
4. Oppenheim, F. G., Xu, T., McMillian, F. M., Levitz, S. M., Diamond, R. D., Offner, G. D., and Troxler, R. F. (1988) Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on Candida albicans. *J Biol Chem* 263, 7472-7477
5. Gusman, H., Lendenmann, U., Grogan, J., Troxler, R. F., and Oppenheim, F. G. (2001) Is salivary histatin 5 a metallopeptide? *Biochim Biophys Acta* 1545, 86-95
6. Yan, Q., and Bennick, A. (1995) Identification of histatins as tannin-binding proteins in human saliva. *Biochem J* 311 (Pt 1), 341-347
7. Oudhoff, M. J., Bolscher, J. G., Nazmi, K., Kalay, H., van 't Hof, W., Amerongen, A. V., and Veerman, E. C. (2008) Histatins are the major wound-closure stimulating factors in human saliva as identified in a cell culture assay. *Faseb J* 22, 3805-3812
8. Lagerstrom, M. C., and Schioth, H. B. (2008) Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat Rev Drug Discov* 7, 339-357
9. Veerman, E. C., Valentijn-Benz, M., Nazmi, K., Ruissen, A. L., Walgreen-Weterings, E., van Marle, J., Doust, A. B., van't Hof, W., Bolscher, J. G., and Nieuw Amerongen, A. V. (2007) Energy depletion protects Candida albicans against antimicrobial peptides by rigidifying its cell membrane. *J Biol Chem* 282, 18831-18841
10. Oudhoff, M. J., van den Keijbus, P. A., Kroeze, K. L., Nazmi, K., Gibbs, S., Bolscher, J. G., and Veerman, E. C. (2009) Histatins enhance wound

closure with oral and non-oral cells. *J Dent Res* 88, 846-850

11. Mochon, A. B., and Liu, H. (2008) The antimicrobial peptide histatin-5 causes a spatially restricted disruption on the Candida albicans surface, allowing rapid entry of the peptide into the cytoplasm. *PLoS Pathog* 4, e1000190
12. Whistler, J. L., Chuang, H. H., Chu, P., Jan, L. Y., and von Zastrow, M. (1999) Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* 23, 737-746
13. Alvarez, V. A., Arttamangkul, S., Dang, V., Salem, A., Whistler, J. L., Von Zastrow, M., Grandy, D. K., and Williams, J. T. (2002) mu-Opioid receptors: Ligand-dependent activation of potassium conductance, desensitization, and internalization. *J Neurosci* 22, 5769-5776
14. Willins, D. L., Berry, S. A., Alsayegh, L., Backstrom, J. R., Sanders-Bush, E., Friedman, L., and Roth, B. L. (1999) Clozapine and other 5-hydroxytryptamine-2A receptor antagonists alter the subcellular distribution of 5-hydroxytryptamine-2A receptors in vitro and in vivo. *Neuroscience* 91, 599-606
15. Simmons, G., Clapham, P. R., Picard, L., Offord, R. E., Rosenkilde, M. M., Schwartz, T. W., Buser, R., Wells, T. N., and Proudfoot, A. E. (1997) Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science* 276, 276-279
16. Mack, M., Luckow, B., Nelson, P. J., Cihak, J., Simmons, G., Clapham, P. R., Signoret, N., Marsh, M., Stangassinger, M., Borlat, F., Wells, T. N., Schlondorff, D., and Proudfoot, A. E. (1998) Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. *J Exp Med* 187, 1215-1224
17. Hoare, S. R. (2005) Mechanisms of peptide and nonpeptide ligand binding to Class B G-protein-coupled receptors. *Drug Discov Today* 10, 417-427
18. Imamura, Y., Fujigaki, Y., Oomori, Y., Usui, S., and Wang, P. L. (2009) Cooperation of salivary protein histatin 3 with heat shock cognate protein 70 relative to the G1/S transition in human gingival fibroblasts. *J Biol Chem* 284, 14316-14325
19. Sun, X., Salih, E., Oppenheim, F. G., and Helmerhorst, E. J. (2009) Kinetics of histatin proteolysis in whole saliva and the effect on bioactive domains with metal-binding, antifungal, and wound-healing properties. *Faseb J* 23, 2691-2701
20. Gautier, R., Douguet, D., Antonny, B., and Drin, G. (2008) HELIQUEST:

a web server to screen sequences with specific alpha-helical properties. *Bioinformatics* 24, 2101-2102

# Chapter

# 7

## Use of sortase A for the efficient and rapid cyclization of long-sized peptides such as histatins

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* equal contribution

Manuscript in preparation

## Abstract

Cyclization of peptides is a commonly used method to impose a certain structural conformation, which will enhance binding affinity and/or activity of the specific peptide. Several techniques are available, but a straightforward and foolproof technique, especially for longer peptides, is not present. The aim of this study was to explore and develop a new technique for the use of cyclization of longer peptides. We wished to use it for the cyclization of the model peptide histatin, a relatively long peptide with wound-healing activities that is originally derived from saliva. The bacterial enzyme sortase was used to cyclisize peptides, and by using sortase we obtained yields above 90 % within 24 h. This is in high contrast with the ~ 2 % yield we obtained with chemical cyclization. We previously found that the chemically-cyclisized histatin has a 1,000-fold higher activity in induction of epithelial-cell migration, and this enhanced activity upon cyclization was confirmed for enzymatic cyclization using sortase. The high yields of the present technique enabled us to demonstrate that a difference in binding affinity for the putative receptor is the basis for the difference between linear and cyclic histatin. In addition, we found possible binding partners at the cellular membrane of epithelial cells, which may be the actual receptor(s) for histatin. Overall, this study establishes the use of sortase for the cyclization of any peptide, so far with remarkable high yields and no demonstrable adverse effects.

## Introduction

Bioactive peptides generally are linear amino-acid sequences that are characterized by a large conformational flexibility. Since the bioactive conformation of the peptide is only one of many that are present in solution, the molar activity of random-structured peptide ligands is lower than proteins or peptides with a more constrained conformation. In our case, enhancement of epithelial-cell migration by the linear peptide Histatin 1 (Hst1) requires concentrations in the μM range, whereas epidermal growth factor (EGF) is similarly active in the nM range (1). Introduction of conformational restraint of peptides through cyclization is a standard but difficult strategy in medicinal chemistry, and it commonly increases receptor affinity and/or selectivity of peptide ligands. Furthermore, cyclization has often been employed as a means of prolonging the duration of action of peptide hormones, because cyclic peptides generally are thermo-stabile, pH-stabile, and less vulnerable for proteolytic cleavage than their linear counterparts (2). These characteristics make them good candidates for drug development (3).

Several chemical strategies for synthesis of cyclic peptides have been described. These include side-chain to side-chain (e.g formation of disulfide bridges between cysteine residues), side chain to terminal group, and terminal group to terminal group (head to tail) cyclizations. Head to tail cyclizations can be accomplished either in solution or while attached to a solid phase resin using some form of chemical coupling to form N-C terminal amides (for a review see (4)). These methods are generally well-suitable for small peptides (5), but are inefficient for cyclization of relative large peptides (larger than 10 residues), because of large entropic barriers for such reactions and competing oligomerization. In addition to the chemical methods, a few enzymatic strategies for cyclization of peptides have been described, such as the use of TycC thioesterase, subtiligase, and intein-based cyclization (6-9).

Recently, enzymatic ligation methods using Sortase A (SrtA) from *Staphylococcus aureus* has been developed for site-specific derivatization of proteins by an array of functional groups, including fluorophores, (photo)affinity labels, and the production of fusion proteins (10-12). In addition, by equipping model proteins C-terminally with the LPXTG-motif and N-terminally with the oligoglycine acceptor motif, they are rapidly and effectively converted by SrtA into the circular form (13). This prompted us to explore the use of SrtA for cyclization of large-sized peptides, which are hardly accessible for cyclization with the present methods. Folded proteins apparently, often have their N- and C-termini in close proximity (2), which is in favour for SrtA-mediated cyclization. For large linear

peptides it is unpredictable how the distance between N- and C-termini may affect SrtA-mediated cyclization, with relative high flexibility favouring, but overall distance potentially disfavouring cyclization.

We previously discovered a large peptide of which the bioactivity drastically is enhanced by cyclization; Hst1, a 38-mer peptide of human saliva which stimulates cell migration in simple and complex wound models (14). Upon cyclization the activating properties are approximately 1000-fold enhanced (14). Further investigation, however, has been hampered by the low synthesis yields (~ 2%) that were reached with the present chemical strategies for cyclization of Hst1. We therefore aimed to develop a new strategy for cyclization of longer peptides using SrtA. We here describe a rapid and efficient method (> 90 % yield) based on SrtA for cyclization of long sized peptides such as Hst1. The biological activity of the enzymatically-cyclized Hst1 was similar to that of chemically-synthesised cyclic Hst1. In competition binding assays was found that the affinity to its cellular receptor was concomitantly increased, and several binding proteins of Hst1 on the cellular membrane were detected.

## Material and Methods

### Solid-phase peptide synthesis

CysHst1(16-34)Cys was obtained from 21st Century Biochemicals (Marlboro, MA). All other peptides (for overview see Table 1) were manufactured by solid-phase peptide synthesis (SPPS) using 9-fluorenylmethoxycarboyl (Fmoc)-chemisty with a MilliGen 9050 synthesizer (MilliGen/Biosearch, Bedford, MA) according to the manufacturer's procedures. Peptide synthesis grade solvents were used directly as obtained from Biosolve (Valkenswaard, The Netherlands). The N-α-Fmoc-amino acids were obtained from OrpegenPharma (Heidelberg, Germany). Preloaded solid phase resin (Fmoc-L-Asn(Trt)-PEG-PS, 0.1 mmol, loading 0.16 mmol/g; Applied Biosystems, Foster City, CA) in 1-methyl-2-pyrrolidone (NMP) was applied to the column and equilibrated at a flow rate of 4 ml/min. Fmoc was removed with piperidine (20 % v/v) in NMP for 6 min. Fmoc-amino acids were dissolved at 4 times molar excess in dimethylformamide (DMF) containing 0.6 mM 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and 0.6 mM 1-hydroxybenzotriazole (HOBt) and coupled in the presence of 0.45 mM N,N-diisopropylethylamine (DIPEA) in NMP by recycling for 1,5 h. Washings between the reaction steps were carried out with NMP. Subsequently the peptide was detached from the resin and deprotected with 14 ml trifluoroacetic acid (TFA)/phenol/thioanisole/$H_2O$ (85:5:5:5) in a 25 ml syringe equipped with a frit, under gentle shaking during at least 2 h. Next $N_2$ was flushed through the reaction mixture to reduce the volume to less than 2 ml. The reaction mixture was purged into 30 ml ice-cold diethylether in a 50 ml tube followed by two washings of the resin with 2 ml TFA. The precipitated peptide was washed 4 times with ice-cold diethylether using a magnetic driven centrifuge (Christ RVC 2-25, Osterode, Germany) at 230 x g for 5 min, dissolved in 10 ml $H_2O$, flushed with $N_2$ to remove excess diethylether and lyophilized.

**Table 1.** Amino acid sequences of peptides used in this study

| Peptides | Amino acid sequence | |
|---|---|---|
| Hst1 | DSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN | |
| cHst1 (chemical) | ~KRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDNDSHE~ | [a] |
| Hst1(16-34) | EKHHSHREFPFYGDYGSNY | |
| CysHst1(16-34)Cys | ~C-EKHHSHREFPFYGDYGSNY-C~ | [a] |
| GG-Hst1-LPETGG | GGDSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDNLPETGG | |
| cGG-Hst1-LPET | ~GGDSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDNLPET~ | [a] |
| GG-Hst1-LPETGG$_{biotin}$ | GGDSHEKRHHGYRRKFHE**K**HHSHREFPFYGDYGSNYLYDNLPETGG | [b] |
| cGG-Hst1-LPET$_{biotin}$ | ~GGDSHEKRHHGYRRKFHE**K**HHSHREFPFYGDYGSNYLYDNLPET~ | [ab] |

[a] ~ residues at N and C termini used for cyclization

[b] **K** biotinylated Lysine residue, incorporated during synthesis

**Peptide purification**

Peptides were purified by semi-preparative RP-HPLC (Jasco Corporation, Tokyo, Japan) on a Vydac C18-column (218MS510, Vydac, Hesperia, CA). Peptides were dissolved in $H_2O$, containing 5% acetonitril (AcN; Biosolve) and 0.1% TFA. Elution was performed with a linear gradient from 15 to 45% AcN containing 0.1% TFA in 20 min at a flow rate of 4 ml/min. The absorbance of the column effluent was monitored at 214 nm, and peak fractions were pooled and lyophilized. Re-analyzed by RP-HPLC on an analytic Vydac C18-column (218MS54) developed with a similar gradient at a flow rate of 1 ml/min revealed a purity of at least 95%. Peptides were routinely verified by ion trap mass spectrometry with a LCQ Deca XP (Thermo Finnigan, San Jose, CA).

**Organic chemical cyclization**

Cyclic Hst1 was synthesized on Fmoc-Glu (Wang resin)-ODmab (NovaBiochem, Läufelfingen, Switzerland). This starting Glu residue was the first of the three Glu residues within the Hst1 sequence resulting in one cyclic peptide cHst1 by a chemical ligation reaction. We tried using the other two Glu residues as well, without success. After completion of the sequence, the N-terminal Fmoc was removed with 20% piperidine in NMP, and subsequently the C-terminal ODmab was removed by 2% hydrazine in DMF. On-resin head-to-tail cyclization was achieved by prolonged reaction (72 h) with 1 eq benzotriazole-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, Biosolve), 1 eq HObt, and 1 eq DIPEA in DMF containing 20% dimethylsulfoxide (DMSO, Biosolve) and 2% dichloromethane (DCM, Biosolve). After cleavage from the resin and purification by RP-HPLC, cyclization was confirmed by MALDI-TOF mass spectrometry, which showed that the molecular mass of cHst1 was 4830 Da, 18 Da less than that of the linear Hst1. The overall yield with either linear peptide as starting peptide was 1 - 3%, which is not unexpected taking into account the length of Hst1 for on-resin synthesis of a cyclic peptide.

**Expression and purification of SrtA**

A soluble version of SrtA was created comprising the catalytic domain of the *Staphylococcus aureus* sortase (amino acids 26 to 206) and a hexahistidine tag at the N-terminus ((18); cloned in to pQE30, Quiagen). Briefly, the Srt A-expression plasmid containing *Escherichia coli* BL-21 (DE3) was cultured in the presence of Ampicillin (10 μg/ml) until $OD_{600}$ ~0.7. SrtA production was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). After an additional 3 h of culturing, bacteria were harvested by centrifugation at 3500 x g at 4 °C for 30

min and resuspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5 containing 150 mM NaCl, 20 mM imidazole, 10% glycerol). Bacteria were lysed by passing through a pre-chilled cell disruption press (One Shot Model, Constant Systems Limited, Daventry Northants, UK) operating at 1250 kpsi. The lysate was cleared by centrifugation at 14,000 x g at 4 °C for 30 min. The supernatants were subjected to affinity chromatography on a recharged $Ni^{2+}$-HisTrap HP column (GE Healthcare, Uppsala, Sweden). The column was washed extensively with lysis buffer containing 50 mM imidazole and eluated with lysis buffer containing 500 mM imidazole. The imidazole was removed by buffer exchange step on a PD-10 desalting column (GE Healthcare). Purity was analyzed by SDS-PAGE and if necessary affinity purification was repeated. The affinity-purified SrtA was stored in 10% glycerol, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl at -80 °C until use. Alternatively, SrtA was purified directly from the HisTrap elution fractions, without buffer changes or imidazole removal, by semi-preparative RP-HPLC, developed with a linear gradient from 25 to 45% AcN containing 0.1% TFA in 20 min at a flow rate of 4 ml/min. HPLC-purified SrtA was lyophilized and stored at -20 °C. The latter method produced SrtA of over 95% purity, which is easy to aliquot at desired concentrations, and stable as dry powder at -20 °C.

**Sortase-catalyzed cyclization and purification of cyclic peptides and SrtA**

Linear peptides subjected to sortase-catalyzed cyclization were equipped with the SrtA cleavage site LPETG at the penultimate position of the C-terminus. To increase the efficiency of the sortase-mediated processing the sortase cleavage site was extended with an additional glycine residue at the C-terminus. A diglycine motif (GG) was appended at the N-terminus which served as a highly efficient acceptor allowing intramolecular ligation by an amide bond. These peptides were synthesized on the solid phase Fmoc-L-Gly-PEG-PS resin (0.1 mmol, loading 0.19 mmol/g; Applied Biosystems), processed and purified as described above. The intramolecular transpeptidation reactions were conducted with 150 μM of the linear peptide and 50 μM SrtA (or otherwise as stated in the legends to the figures) in sortase reaction buffer (50 mM Tris, pH 7.5, containing 150 mM NaCl and 10 mM $CaCl_2$) at 37 °C. The reaction was monitored by analysis of samples by RF-HPLC on an analytical C18 column (218MS54, Vydac) developed with a gradient from 30 to 45% AcN in 20 min at a flow rate of 1 ml/min. After the reaction was completed the reaction mixture was separated by semi-preparative RP-HPLC on a Vydac C18 column (218MS510), eluted with a gradient from 25 to 40% AcN containing 0.1% TFA in 20 min at a flow rate of 4 ml/min. This gradient allowed separation of the

cyclic peptide from the linear remnants and from SrtA. Fractions containing the cyclic peptide and SrtA, respectively, were pooled, lyophilized and stored as dry powder at -20 °C.

**SrtA activity on FRET-based substrate**

SrtA activity was monitored using Fluostar Galaxy microplate fluorimeter (BMFG Labtechnologies, Offenburg, Germany) using a FRET bacterial sortase substrate I (AnaSpec, Fremont, CA, USA) according to manufacturer. Fluorescence was determined per mg SrtA.

**MS/MS sequencing of proteolytic fragments from cyclic Hst1**

Purified cyclic Hst1 (cGG-Hst1-LPET) was subjected to trypsin digestion. The peptides generated from proteolytic digestion were extracted and concentrated for analysis by RP-HPLC and tandem mass spectrometry. RP-HPLC was carried out on a Waters NanoAcquity HPLC system with a flow rate of 250 nl/min and mobile phases of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The gradient used was isocratic 1% acetonitrile for 1 min followed by 2% acetonitrile per min to 40% acetonitrile. The analytical column was 0.075 μm × 10 cm with the tip pulled to 0.005 μm and self-packed with 3 μm Jupiter C18 (Phenomenex). The column was interfaced to a Thermo LTQ linear ion trap mass spectrometer in a nanospray configuration, and data were collected in full scan mode followed by MS/MS analysis in a data-dependent manner. The mass spectral data were data base searched using SEQUEST.

**Wound-closure assay**

*In vitro* wound closure experiments were performed in 48 wells culture plates (Greiner-Bio-One, Alphen a/d Rijn, The Netherlands) with human buccal epithelial cells (HO-1-N-1, provided by the Japanese Collection of Research Bioresources, Osaka, Japan). With a sterile tip a scratch was made in a confluent layer of cells that were serum-deprived in DMEM/F12 culture medium (Invitrogen, Carlsbad, CA) for 24 h. The width of the scratch was measured at the beginning and after 18 h of culture on microscopic images (Leica DM IL, Leica DFC320 camera; Leica Microsystems, Wetzlar, Germany). Relative closure was calculated by dividing the closure in the presence of peptide by that in the absence of peptide. Final concentration of synthetic peptides in the assays varied between 0.1 nM and 10 μM as indicated. As a positive control, 10 ng/ml rhEGF (Invitrogen) was used.

**Peptide-binding assay**

Peptide binding to the cell surface was performed with HeLa cells. Scraped cells at a $10^6$ cells/ml density were incubated with different concentrations of linear, cyclic, biotinylated, and unbiotinylated Hst1 variants. As proof for Hst-specific binding, controls containing also an excess of unbiotinylated Hst were included. Incubations were performed at 4 °C to prevent uptake of peptides. After 1 h cells were washed three times in cold PBS, and lysed with 50 mM Tris (pH = 8), 150 mM NaCl containing 1% SDS. The lysate was incubated with 20 µl streptavidin beads (Pierce) for 30 min at RT. Beads were collected by centrifugation, washed three times with PBS containing 0.1% Tween-20 and boiled in SDS-PAGE loading buffer to dissociate the biotin-avidin complexes. After centrifugation the supernatants were analysed by SDS PAGE (12% Tris-Trycine), followed by Western blotting on nitrocellulose, blocked 5% BSA PBS-T for 30 min, and 30 min incubation with 1:5000 streptavidin-HRP, signal was detected with ECL kit (Pierce).

**Receptor isolation using [35S]Met/Cys labelled HeLa cells**

Cells were grown to confluence on a 15 cm diameter dish, washed once with PBS, and incubated with 10 ml [35S]Met/Cys (250 µCi/ml) for 4 h. Cells were scraped and a binding assay at 4 °C was performed with three conditions; i) 10 nM cyclic-biotinylated cHst1, ii) 10 nM cyclic-biotinylated cHst1 + 10 µM cyclic-unbiotinylated cHst1, iii) control without peptide. Then, cells were lysed with 0.4% NP40, 0.1% SDS, in PBS for 1 h at 4 °C, and the lysate was centrifuged to remove nuclei and DNA. Supernatant was incubated with 10 µl Streptavidin beads (Pierce) in 0.1% SDS in PBS. Beads were spun down and washed three times with ice-cold PBS with 0.1% SDS. Hst1-binding proteins were specifically eluted from the beads using 10 µM unbiotinylated cHst1. Supernatant was dissolved in loading buffer, boiled, and loaded on a 10% acrylamide (SDS-PAGE), which was run overnight at 50 V. The gel was washed three times with DMSO, and fixed in 22% PPO (2,5-Diphenyloxazole) in DMSO, and then washed for 1 h with water. The polypeptides on the gel were visualized using fluorography.

## Results

### SrtA-mediated cyclization of Hst1 is fast and efficient

In a previous study, we cyclisized Hst1 using a conventional on-resin method (14). To compare this technique with SrtA-mediated cyclization we show here first the results of the reaction process of the on-resin method. Figure 1A shows a schematic overview of the synthesis of cyclic Hst1 with conventional on-resin cyclization method. After synthesis the final product was lyophilised and analysed by HPLC (Fig. 1B), which revealed the presence of a heterogeneous mixture of numerous molecular species. By MS analysis of all fractions we identified the presence of peptides with molecular masses corresponding to linear and cyclic Hst1, respectively, in two minor peaks. The peak containing the putative cyclic hst1 represented approximately 2% of the final material, the bulk of the synthesized material included many unknown peptides presumably including di- and trimers (data not shown). Cyclization using the other two Glu residues in the Hst1 sequence as starting point showed no better results (data not shown). Our results are illustrative for the difficulty and unpredictability of conventional on-resin cyclization method for long peptides, and underscore the pressing need for a new technique. From these peaks we isolated a cyclic variant with the appropriate mass and having a slightly longer retention time on HPLC than its linear variant (Fig. 1C). The low yields made it very difficult for us to initiate follow-up studies on our initial finding that the cyclic variant is 1,000-fold more active, which prompted us to explore other possible cyclization methods. The first possibility to resolve the low-yield problem was to try cyclization by sulphur bridges of two Cys residues, although this method limits the peptide length to approximately 20 residues. We obtained this, in our case 21-mer including two Cys residues, commercially and tested its activity in the cell-migration assay. However, this cyclic Hst (CysHst1(16-34)Cys) did not show the 1000-fold increase in activity; actually it was even less active than its linear counterpart without Cys residues (Hst1(16-34)) (Fig. 3C).

Since SrtA has been used for efficient cyclization of model proteins (13), we explored its use for synthesis of cyclic Hst1. This requires extention of Hst1 at the C-terminus with LPETGG, and at the N-terminus with GG (GG-Hst1-LPETGG). Sortase cleaves the amide bond between Thr and Gly in the LPETGG motif, generating a covalent acyl-enzyme intermediate. Subsequent nucleophilic attack by the di-glycine motif at the N-terminus will give rise to an intra-molecular peptide bond between the N-terminal Thr and the C-terminal Gly, resulting in a head to tail cyclic peptide (Fig. 2A). We incubated GG-Hst1-LPETGG (0.5 mM) with SrtA (50 μM) in sortase reaction buffer and monitored the reaction by RP-HPLC and

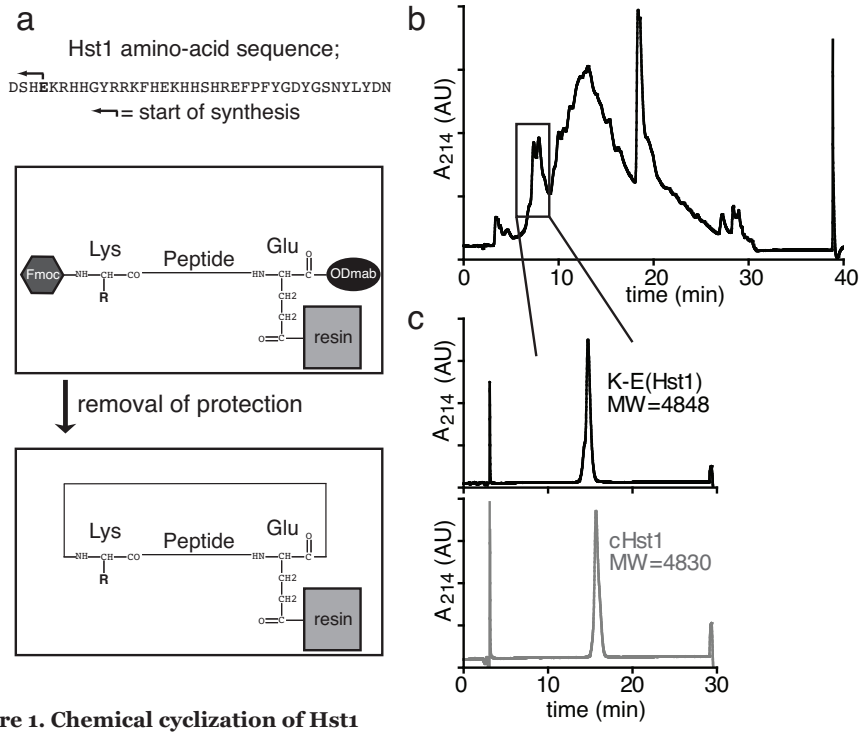



**Figure 1. Chemical cyclization of Hst1**

**A** Illustration of the chemical cyclization of Hst1. Glutamic acid at position 4 was used as starting point for peptide synthesis, whereas normally the N-terminal Asn is used for this. The side chain was attached to the resin and the peptide was synthesized as depicted in the upper panel. When peptide synthesis was completed the protection sites were removed so that free reaction could occur, including cyclization, as depicted in the lower panel. **B** RP-HPLC profile after synthesis. The two peaks depicted in the box were collected purified and pooled. The large bulk of peaks that have longer retention time contains all kinds of by-products, MS analysis revealed large constructs, probably dimers and trimers. **C** RP-HPLC profiles of isolated fractions depicted in box in B, separated on an analytical column (acetonitile gradient 5-30 %). Linear K-E(Hst1) thus has a different sequence compared to Hst1 (see A). Linear Hst1 has a slightly shorter retention time compared to cHst1. MALDI-TOF analysis revealed corresponding molecular weights with the difference of a lactam bond.

SDS-PAGE (Fig. 2B & C). After 21 hr more than 90 % of the starting material was converted into virtually one product with a slightly longer retention time on HPLC (Fig 2B). ESI-MS analysis confirmed that this was comprised of one single species with a molecular mass of 5384, corresponding to that of the expected circular product (cyclic GG-Hst1-LPET) (Fig. 2D). ESI-MS analysis of trypsin-treated cyclic GG-Hst1-LPET revealed the presence of fragment DN**LPETGG**DSHEKRHHGY, confirming that transpeptidation between the C-terminal LPET and the N-terminal GG-motif indeed had occurred (Fig. 2E). In addition to different Hst variants, we also cyclisized other peptides with sizes varying from 12 to 27 residues (SDF-1-derived, RANTES-derived) with the same yield and ease (data not shown).

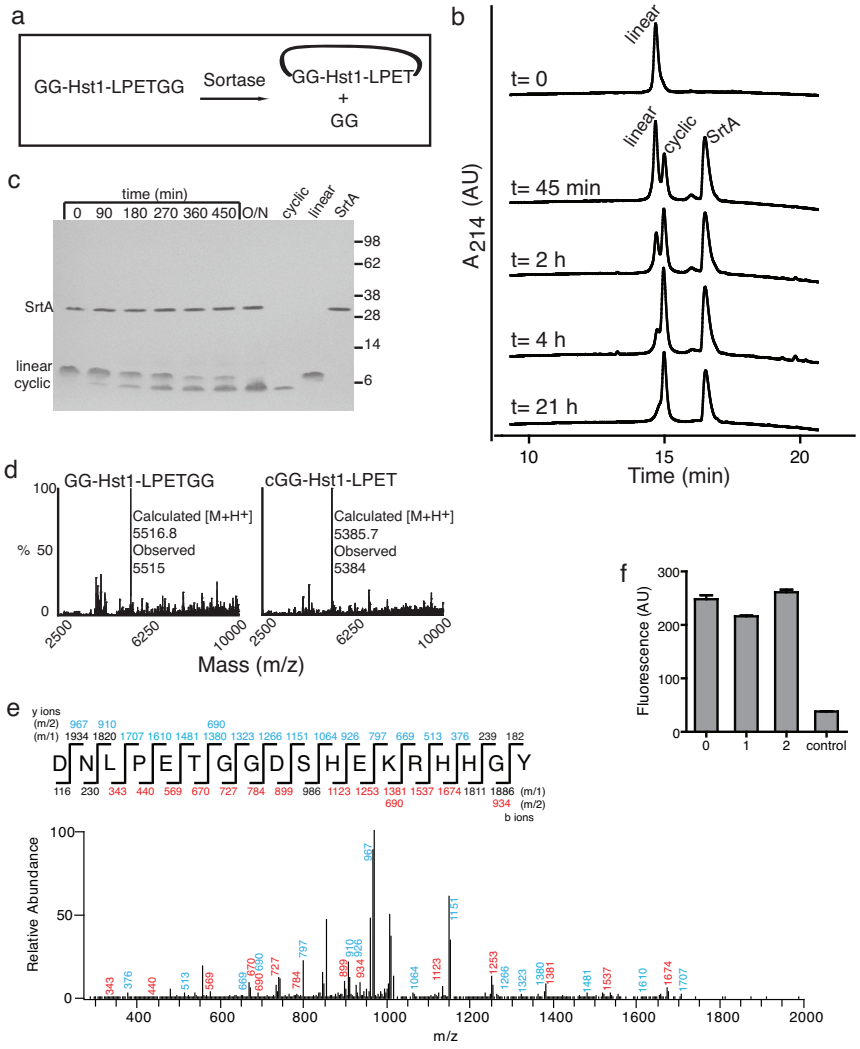
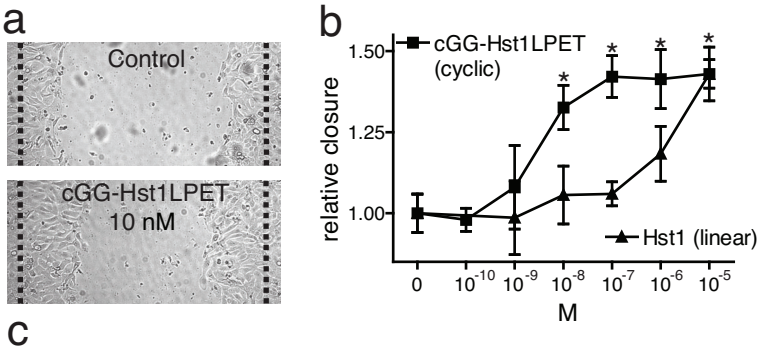



**Figure 2** SrtA-mediated cyclization of Hst1

**A** Schematic illustration of enzymatic cyclization of Hst1 variant using the bacterial enzyme SrtA. **B** RP-HPLC profiles of the reaction of SrtA-mediated cyclization of GG-Hst1-LPETGG (linear) to cGG-Hst1-LPET (cyclic) at different time points. **C** Coomassie-stained SDS-PAGE gel of the cyclization reaction. In control lanes purified cGG-Hst1-LPET (cyclic), GG-Hst1-LPETGG, and SrtA is loaded. **D** MALDI-TOF analysis of GG-Hst1-LPETGG and cGG-Hst1-LPET of RP-HPLC-separated fractions. Differences in mass correspond to predicted differences between cyclic and linear versions. **E** MS/MS spectrum of a tryptic fragment of cGG-Hst1-LPET showing the ligation of the N-terminal residues (GGDSHEK) to the C-terminal LPET motif. Expected masses for y and b ions are listed *above* and *below* the peptide sequence. Ions that were positively identified in the MS/MS spectrum are highlighted in *blue* or *red*. The most prominent daughter ions have been labeled in the MS/MS spectrum. **F** SrtA activity measured with FRET peptide-substrate. SrtA activity (1 mg/ml) immediately after isolation (0), used once for cyclization of Hst, pooled fraction collected by RP-HPLC, lyophilized and dissolved in reaction buffer at 1 mg/ml (1), used twice as described above (2), and control is substrate alone.

Figure 2B shows that the SrtA containing peak, migrating at much longer retention time, was completely separated from the peptides. We pooled this peak to test whether SrtA maintain its enzymatic activity after lyophilisation and reconstitution in buffer. Enzymatic activity, measured using a FRET-based substrate, appears almost completely recoverable, even after three HPLC/ lyophilisation cycles (Fig 2F). This makes it possible to re-use SrtA.

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| Peptide | Active conc. (M) | Yield |
|---|---|---|
| Hst1 | $10^{-5}$ | - |
| cHst1 (chemical) | $10^{-8}$ | ~ 2 % |
| Hst1(16-34) | $10^{-5}$ | - |
| CysHst1(16-34)Cys | $10^{-4}$ | commercial |
| GG-Hst1-LPETGG | $10^{-5}$ | - |
| cGG-Hst1-LPET | $10^{-8}$ | > 90 % |
| GG-Hst1-LPETGG$_{biotin}$ | $10^{-5}$ | - |
| cGG-Hst1-LPET$_{biotin}$ | $10^{-8}$ | > 90 % |

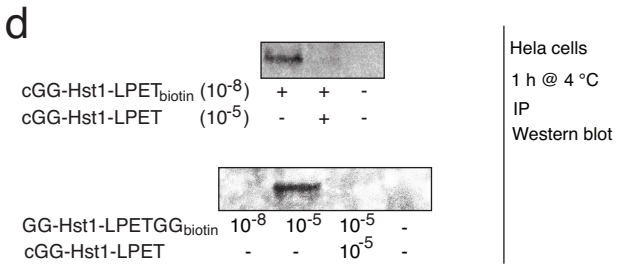


**Figure 3** Activity and binding affinity of cyclic and linear Hst variants

**A** Example of wound closure activities in of cGG-Hst1LPET (10 nM) in confluent HO-1-N-1 cells. Dotted line represents 'wound' prior incubation, pictures are representative after 18 h of incubation. **B** Dose-response curves of wound closure experiments such as illustrated in (A) comparing Hst1 and cGG-Hst1LPET. n=18, means ± SD. **C** Minimal concentrations needed for maximal activation of the peptides used in this study, as can be deducted from dose-response curves shown in (B) from the assays as illustrated in (A). In addition, the yields of cyclization of the different peptides as can be deducted from RP-HPLC profiles as shown in figure 2B. **D** Binding studies with biotinylated cyclic and linear Hst variants. Upper panel: cGG-Hst1-LPET$_{biotin}$ binds to HeLa cells enough for detection at $10^{-8}$ M, this can be nullified upon competition with cGG-Hst1-LPET at $10^{-5}$ M, thus a 1:1000 ratio. This is indicative for Hst-specific binding to HeLa cells. Lower panel: GG-Hst1-LPETGG does not bind sufficiently for detection at $10^{-8}$ M but does at $10^{-5}$ M. This binding can be abolished with cGG-Hst1-LPET in a 1:1 ratio at $10^{-5}$ M. This indicates that cyclic and linear have the same binding partner at the cell surface, but that cyclic Hst1 has a ~ 1,000-fold higher affinity for it.

We determined the biological activity of SrtA mediated cyclic Hst1 (cGG-Hst1-LPET) in a cell migration scratch assay (Fig. 3A). Dose response curves show a 1.000-fold higher activity of the cyclic variant compared to the linear variant (Fig. 3B). Thus, the additional LPETGG motif demanded by SrtA does not affect the biological activity. Also, the biotinylation of Hst1, which was needed for binding studies (see below), formed no hurdle for the cyclization, and the activity of the biotinylated cyclic Hst1 is equal to that of the unbiotinylated (Fig. 3C).

**Cyclization increases the affinity for the receptor**

We hypothesized that the enhanced activity of the cyclic variant was due to higher binding affinity for the cellular receptor. To test this we performed competition binding studies with different linear and cyclic Hst1 variants. Use of anti-Hst antibodies for monitoring binding of a specific variant, however, was not feasible because these recognize both the linear and the cyclic form. Therefore we made cyclic versions of biotinylated Hst1 using the developed sortase-based cyclization method. As mentioned above, biotinylation did not affect the activity of Hst1 (Fig. 3C). Hela cells were incubated with 10 nM of cyclic-biotinylated Hst1 at 4 °C with and without 10 μM of unbiotinylated cyclic Hst1. Cell-associated biotinylated Hst1 was detected by Western blotting using avidin-peroxidase detection (Fig 3D). At the lowest concetration tested (10 nM), binding of cyclic Hst1, but not of linear Hst1, occurred. This binding was prevented in the presence of 10 μM unlabeled cyclic Hst, indicating that it was not mediated by the biotin-moiety of the peptide. Binding of the biotinylated linear variant occurred only at a concentration of 10 μM. This binding was absent when incubation was conducted in the presence of 10 μM biotinylated cyclic Hst1. This suggests that both variants compete for the same receptor. Overall the results of these binding studies support that the concept that constraining the conformational freedom of Hst1 stabilizes the bioactive conformation that is recognised by the receptor.

**Candidate receptors identified using cyclic biotinylated Hst1**

To identify the cellular receptor for Hst1 we used cyclic-biotinylated Hst1 as high-affinity ligand for affinity purification. Hela epithelial cells, metabolically labelled with [35S]Met/Cys, were incubated with biotinylated-cyclic Hst1 for 1 h, at 4 °C to prevent internalization of the ligand-receptor complex. As control the same incubations were done with 1000-fold excess unbiotinylated-cyclic Hst1, and with medium alone. Cells were lysed and proteins extracted as described in the material and methods. Streptavidin-coated beads were added to the protein extract to precipitate biotinylated protein (complexes). Although several precautionary

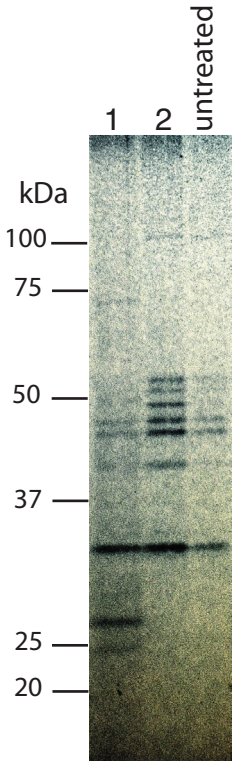


**Figure 4.** Identification of binding partners of Hst1 at the cellular membrane using radioactive labelled HeLa cells.

Hela cells were labelled with [35S]Met/Cys for 4 h, and treated with 10 nM cGG-Hst1-LPET$_{biotin}$ (1), 10 nM cGG-Hst1-LPET$_{biotin}$ and 10 µM cGG-Hst1-LPET (2), or untreated at 4 °C for 1 h. Cells were lysed and biotinylated protein (compexes) were immune-precipitated with Streptavidin beads. Subsequently, Hst-binding proteins were eluted from the beads with 10 µM cGG-Hst1-LPET, and supernatants were loaded and separated by SDS-PAGE (10% acrylamide). Around 30 and 70 kDa unique bands are visible in lane 1, indicating possible binding partners of Hst1.

measures were taken, we still observed a lot of background when eluting proteins from the beads by boiling. To enhance specificity, the beads were eluted with unbiotinylated Hst1, to specifically dissociate histatin-receptor complexes. In this way only proteins bound via a (biotinylated)histatin ligand will be released into solution, whereas other complexes (e.g. between avidin binding proteins) remain linked to the beads. The supernatant was separated with gel electrophoresis, and after development for 24 h unique radioactive bands could be detected at approximately 30 kDa and 70 kDa in the samples obtained after incubation of cells with cyclic biotinylated Hst1 (Fig. 4). These bands were not visible in both control lanes, indicating that they are binding proteins for Hst1 at the cellular membrane of epithelial cells.

## Discussion

Cyclization of peptides is a common method to optimize activity or binding, and it protects peptides against degradation enhanced by pH or heat, or against proteolysis due to exo-proteases. These are all characteristics that could be important for drug development, but also provides extra means for characterization of molecular mechanisms of action. Chemical cyclization methods are feasible for small peptides, but have to be optimized for every individual peptide. In addition to chemical cyclization, several enzymatic cyclization methods have been developed, but most of them still have restrictions. In this study we used SrtA, a bacterial enzyme that covalently links proteins to the cell wall, to cyclisize Hst1. The average yields were well over 90 % and the reaction was routinely completed within 24 h. Cyclic Hst1 is 1,000-fold more active than its linear analogue, which is attributed to enhanced binding affinity of the cyclic variant vs. the linear one. We found more than one binding proteins of Hst1 at the epithelial cellular membrane, which could be the receptor through which Hst1 induces cell migration.

There are several other enzymatic peptide cyclization methods. The biotechnological use of the thioesterase domain of tyrocidine synthetase was promising for its own specific target with yields around 85 % (7). But, apparently it requires a certain structural conformation of a peptide, as other peptides clearly have much lower yields (~15%) (8). In addition, this technique seems to be restricted for small peptides, thus in contrast to SrtA-mediated cyclization. The split-intein technique for cyclization of peptides is a successful technique for high-throughput of randomized peptides (15-17), and is also used for larger peptides and proteins (6). But this always requires the expression of the requested peptide sequence by an organism, commonly *E. coli*. In our opinion, this is thus inconvenient compared to the current standard SPPS in which peptides can easily be altered by biotinylation, or fluorescent labelling. For biotechnological development this is more convenient since it expands the possibilities. Moreover, in comparison to split-intein technique, SrtA requires only a relatively small modification of the peptide or protein (13). Subtiligase has also been used to cyclisize longer peptides of minimally 12 amino acids, with a maximal yield of 85 % (9). However, despite the apparent feasibility of this enzyme-based method, relatively few studies have appeared in which it has been used. Overall, the use of SrtA for the cyclization of peptides and proteins is, so far, very promising and is unmatched by the other techniques as far as we know. The SrtA cyclisized peptides will, however, contain an additional LPXTG motif that could have an effect on activity. In our case, this did not alter activity, probably because the active motif of Hst1 is not near the LPXTG motif.

For our search for the molecular working mechanism behind Hst-mediated cell activation, the use of SrtA has been a powerful tool. Our current study can easily be followed-up and expanded. Comparable binding experiments can be conducted with a wide variety of Hst variants to ultimately pinpoint the specific residues in the peptide. In addition, our explorative experiments to search for the receptor demonstrated more than one Hst-binding protein on the cellular membrane, out of which the receptor possibly can be isolated after scaling-up the isolation procedures.

## Acknowledgements

This work was supported by a Journal of Cell Science Travelling Fellowship and an EMBO Short-term fellowship (ASTF 86-2010). E.C.I. Veerman gratefully acknowledges the support from the Skeletal Tissue Engineering Group Amsterdam (STEGA).

## References


1. Oudhoff, M. J., Bolscher, J. G., Nazmi, K., Kalay, H., van 't Hof, W., Amerongen, A. V., and Veerman, E. C. (2008) Histatins are the major wound-closure stimulating factors in human saliva as identified in a cell culture assay. *Faseb J* 22, 3805-3812
2. Trabi, M., and Craik, D. J. (2002) Circular proteins--no end in sight. *Trends Biochem Sci* 27, 132-138
3. Namjoshi, S., and Benson, H. A. (2010) Cyclic peptides as potential therapeutic agents for skin disorders. *Biopolymers*
4. Lambert, J. N., Mitchell, J. P., and Roberts, K. D. (2001) The synthesis of cyclic peptides. *Journal of the Chemical Society, Perkin Transactions 1*, 471
5. Harrison, R. S., Shepherd, N. E., Hoang, H. N., Ruiz-Gomez, G., Hill, T. A., Driver, R. W., Desai, V. S., Young, P. R., Abbenante, G., and Fairlie, D. P. (2010) Downsizing human, bacterial, and viral proteins to short water-stable alpha helices that maintain biological potency. *Proc Natl Acad Sci U S A* 107, 11686-11691
6. Xu, M. Q., and Evans, T. C., Jr. (2001) Intein-mediated ligation and cyclization of expressed proteins. *Methods* 24, 257-277
7. Trauger, J. W., Kohli, R. M., Mootz, H. D., Marahiel, M. A., and Walsh, C. T. (2000) Peptide cyclization catalysed by the thioesterase domain of tyrocidine synthetase. *Nature* 407, 215-218
8. Kohli, R. M., Takagi, J., and Walsh, C. T. (2002) The thioesterase domain from a nonribosomal peptide synthetase as a cyclization catalyst for integrin binding peptides. *Proc Natl Acad Sci U S A* 99, 1247-1252
9. Jackson, D. Y., Burnier, J. P., and Wells, J. A. (1995) Enzymic Cyclization of Linear Peptide Esters Using Subtiligase. *Journal of the American Chemical Society* 117, 819
10. Proft, T. Sortase-mediated protein ligation: an emerging biotechnology tool for protein modification and immobilisation. *Biotechnol Lett* 32, 1-10
11. Popp, M. W., Antos, J. M., and Ploegh, H. L. (2009) Site-specific protein labeling via sortase-mediated transpeptidation. *Curr Protoc Protein Sci* Chapter 15, Unit 15 13
12. Popp, M. W., Antos, J. M., Grotenbreg, G. M., Spooner, E., and Ploegh, H. L. (2007) Sortagging: a versatile method for protein labeling. *Nat Chem Biol* 3, 707-708
13. Antos, J. M., Popp, M. W., Ernst, R., Chew, G. L., Spooner, E., and Ploegh, H. L. (2009) A straight path to circular proteins. *J Biol Chem* 284, 16028-16036
14. Oudhoff, M. J., Kroeze, K. L., Nazmi, K., van den Keijbus, P. A., van 't Hof, W., Fernandez-Borja, M., Hordijk, P. L., Gibbs, S., Bolscher, J. G., and Veerman, E. C. (2009) Structure-activity analysis of histatin, a potent wound healing peptide from human saliva: cyclization of histatin potentiates molar activity 1,000-fold. *Faseb J* 23, 3928-3935
15. Scott, C. P., Abel-Santos, E., Wall, M., Wahnon, D. C., and Benkovic, S. J. (1999) Production of cyclic peptides and proteins in vivo. *Proc Natl Acad*

*Sci U S A* 96, 13638-13643
16. Scott, C. P., Abel-Santos, E., Jones, A. D., and Benkovic, S. J. (2001) Structural requirements for the biosynthesis of backbone cyclic peptide libraries. *Chem Biol* 8, 801-815
17. Tavassoli, A., and Benkovic, S. J. (2007) Split-intein mediated circular ligation used in the synthesis of cyclic peptide libraries in E. coli. *Nat Protoc* 2, 1126-1133
18. Ton-That, H., Liu, G., Mazmanian, S. K., Faull, K. F., and Schneewind, O. (1999) Purification and characterization of sortase, the transpeptidase that cleaves surface proteins of Staphylococcus aureus at the LPXTG motif. *Proc Natl Acad Sci U S A* 96, 12424-12429

# Chapter 8

# General discussion

## Histatins

Histatins are a class of histidine-rich peptides that occur exclusively in saliva of humans and primates (1). In human saliva at least 26 histatin peptides have been found (2), although the general consensus is that three members histatin 1 (Hst1), Hst3, and Hst5 comprise about 85 % of the total histatin concentration of saliva (3). All histatins are derived from two closely related genes *HTN1* and *HTN3*, which give rise to two parent peptides Hst1 and Hst3. These two peptides in turn, due to proteolytic cleavage, give rise to the rest of the histatins. Interestingly, Hst1 is merely the parent peptide of one descendant, Hst2, whereas Hst3 gives rise to all the other histatin peptides in human saliva.

Ever since the first description of 'histidine-rich peptides' (the name histatin was introduced a decade or so later) in human saliva in 1976 (4), a large repertoire of different functions have been attributed to them (summarized in Table 1), including:

- Protection against demineralization of the dental enamel, based on the fact that they bind to hydroxyapatite *in vitro* as well as *in vivo*, and their presence in the pellicle (5, 6).
- Inhibition of hydroxyapatite crystal formation (7), suggesting that it plays a role keeping saliva supersaturated with respect to calcium-hydroxyapatite, the mineral phase of dental enamel.
- Detoxification of noxious foodstuffs, based on tannin-binding properties (8, 9).
- Suppression of inflammatory induction of IL-6 and IL-8, due to their LPS-neutralizing properties (10, 11)
- Protection against excessive proteolytic degradation of oral tissues by inhibiting host and bacterial proteases (12-16).
- Protection against bacterial and fungal infections. Because histatins may combine a net positive charge with hydrophobic residues and the ability to adopt an amphipathic conformation, they have the propensity to insert into the phospholipid phase of microbial membranes, leading to leakage of vital cell components (17). Histatins were among the first antimicrobial peptides that were discovered (18-20)

The affinity of histatins for such a large repertoire of chemically and structurally different ligands is obviously linked to the chemical and structural features of this molecule. For example, Hst5 molecules have a flexible structure: in water they have a random-coil structure, while in apolar media they can adopt an $\alpha$-helical structure. This structural flexibility, in addition to the presence of both charged

and hydrophobic domains, is probably the key to the multi-binding features of histatins, as it will favour binding to structurally and chemically widely different molecules. It has to be noted that the above-mentioned functional properties are not very specific. Binding to hydroxyapatite, neutralization of LPS, and disruption of phospholipid double layers all are properties that histatins share with a large number of structurally unrelated, often positively charged amphipathic peptides and proteins. Telling in this respect is the fact that the all-D-enantiomer of Hsts exhibit similar properties, underlining that these binding properties do not involve stereo-isomer-specific ligand-receptor interactions which are so characteristic for biological systems.

The antifungal properties have been studied most, with its most potent member Hst5, being the subject in the majority of these studies. However, several groups have casted doubt on the antimicrobial function of histatins in saliva, because the ability of histatins to kill microbes is salt sensitive (**Chapter 2,** (21)). Saliva is a complex solution with an ionic strength of approximately 50 mM, a concentration at which histatins are virtually inactive against microbes. The abovementioned makes this role of histatins in saliva quite doubtful. The antimicrobial function is caused by the properties of histatins, which are similar, albeit milder in both charge and amphipaticity, to other antimicrobial peptides (**Chapter 5**).

**Table 1.** An overview of the different properties and functions of histatins except for wound healing.

| Properties | Proposed function | Functional group | References |
|---|---|---|---|
| Enamel binding (pellicle protein) | Remineralization, Protection of teeth | Phosphate group (Hst1) | (5, 7, 22, 23) |
| Tannin binding | Detoxification | Arg, Lys, and His residues | (8, 9) |
| Metal binding | Antimicrobial, Inhibitor metalloproteases | His residues and specific motifs for metal-ion binding | (14-16, 24-27) |
| Membrane disturbing | Antimicrobial | Positive charge and amphipaticity | (3, 17, 28, 29) |
| Polysaccharide binding | Immune-modulation | Positive Charge | (10, 11) |
| Protease binding | Protease inhibitor | Competition substrate, metal binding | (12-16) |

## Cell activation by histatin: have we finally found its real biological function?

Because of the many activities displayed by histatins, they often have been considered as multifunctional peptides, involved in a variety of physiological processes in the mouth. In this thesis yet another functional property of histatins is revealed, the stimulation of migration of epithelial cells and fibroblasts. In this respect histatins somewhat resemble chemokines, a totally different family of polypeptides, which mediate chemotactic and chemokinetic responses in different cell types, including epithelial cells, fibroblasts, and neutrophils. The question now arises: is this chemokine-like activity the real biological function of histatins, or is it yet another property that can be added to a long list? A definite answer to this question is hard to give, yet this activity of histatin displays a number of features both on the molecular and functional level that are clearly distinct from the other activities attributed to histatins (Table 1).

### Molecular structural aspects

As stated above, the previously discovered activities of histatins are mainly driven by relatively a-specific physico-chemical interactions, which do not require the presence of a strictly defined complementary three-dimensional conformation, but rather depend on a more global, spatial distribution of positively charged and hydrophobic domains in the peptide. In a similar vein as the membranolytic activity, the LPS-neutralizing immuno-modulatory properties of histatins are based on binding to bacterial LPS, which is driven by electrostatic Coulomb-attraction between the two oppositely charged molecules, in combination with Van der Waals-interactions with the lipophilic fatty acid tails of membrane phospholipids and LPS, respectively. This property is shared with a large number of unrelated amphipathic cationic peptides that bind and neutralize LPS, including LL-37 (30-32), NK-lysin (33), and indolicidin (34). Similarly, binding to hydroxyapatite is not a histatin-specific property, as hydroxyapatite is widely used in preparative biochemistry as chromatographic medium for the fractionation and purification of antibodies, enzymes, and nucleic acids.

The only property of histatins described hitherto that seemingly not can be directly predicted from their chemical structure is their ability to inhibit bacterial and host proteinases (13, 14, 16, 35). However, there has been cast some doubt on whether histatins indeed are bona fide protease inhibitors. It has been demonstrated that the inhibitory activity of histatins gradually decreases during incubation with proteinases, because they are enzymatically degraded. In other words, the

inhibitory activity of histatins is due to competition with other substrates (12). Histatin also *in vitro* inhibits metallo-proteinases, which has been attributed to their metal-binding properties (15). Histidine-rich peptides have strong affinity for metal ions, it is therefore not unexpected that histatins act as inhibitors of metallo-proteinases requiring metal ions as cofactor for their enzymatic activity.

In contrast to these more or less aspecific activities, histatin-mediated activation of cell-migration involves stereospecific binding to a protein receptor which requires a proper three-dimensional conformation of histatin (**Chapters 2, 3, 4, and 7**).

**Functional aspects**

Many previously described functions of histatin, such as detoxification of tannins, neutralization of LPS, and protease inhibition, deal with down-regulation of already existing functions of another biologically active compound or system, rather than with an active role for histatins. It is conceivable that (non-specific) complexation to biomolecules can be sufficient to alter their functional activity. In contrast, the cell-stimulating 'chemokine-like' activity of histatin (**Chapters 2 and 4**) is a biological activity that involves upregulation of specific intracellular processes, and serves a physiologically relevant function.

Another difference is that the previously described 'classical' functions of histatins all have been postulated on the basis of the properties found *in vitro* for isolated histatins, either purified from parotid saliva, or produced by chemical synthesis or recombinant expression. Hence, it has not always been possible to reproduce these activities in more complex systems, such as saliva. A striking example is the salt-sensitivity of the antimicrobial activity of histatins, mentioned above. Although, this by itself does not disprove their physiological significance, it illustrates a problem which is inherent to the approach of trying to find a biological relevant property or activity starting from the isolated compound. On the other hand, the newly found cell-activating function of histatins was detected in an activity assay by stepwise fractionation of fully active saliva to narrow down the active component. Starting with the observation that saliva displayed cell-activating properties, we ended up with the identification of the responsible constituent, which happened to be histatin. These arguments do not present a watertight proof, but they consistently point towards a role for histatin as a bona fide signalling molecule with chemokine-like properties.

**Mechanistic aspects of the histatin-induced cell-activation**

Although histatin shares no structural homology with other known cell signalling polypeptides (chemokines, growth factors), the interaction with its target cell possesses all the characteristics of a classical ligand-receptor mediated process:

- It is a stereo-specific interaction with a putative receptor on the cell membrane. Activity seems to be conveyed by a well-defined three-dimensional conformation, since constraining the conformational freedom of the linear peptide enhanced the activity tremendously (**Chapters 4 and 7**). Paradoxically, by NMR analysis no structural differences between the linear and cyclic form could be detected (unpublished data).
- The histatin-mediated activation is specifically abrogated by an inhibitor of MEK1/2, which is a key-regulator in numerous receptor-mediated cell-signaling processes (**Chapter 2**).
- Histatin-induced cell migration is inhibited by pertussis toxin, a poison that selectively blocks the activation of G-protein by a specific class of receptors, the G-protein-coupled receptors (GPCRs). Sensitivity to PTx thus is considered a specific hallmark of GPCR-dependent signalling processes (**Chapter 4**).
- Histatin is taken up by the cell, in an energy-dependent, temperature-sensitive process, requiring a trypsin-sensitive receptor. Endocytosis of ligand-receptor complexes is a well-recognized step in the signalling process, by which cells are desensitized for signalling (**Chapters 2 and 6**).

In view of these characteristics we postulate that histatins function as ligands for a GPCR on human epithelial cells and fibroblasts. GPCRs comprise a large family of trans-membrane receptors that interact with extra-cellular molecules: GPCR-molecule binding triggers the activation of intracellular signal transduction pathways that ultimately result in a cellular response. The ligands that bind and activate GPCRs include odors, light-sensitive compounds, hormones, and neurotransmitters, and these ligands vary in size from small molecules to peptides to large proteins. Sequencing of the human genome identified 700 to 1000 genes that encode GPCRs, including at least 175 “orphan” receptors. Orphan receptors possess the seven trans-membrane spanning domains typical of GPCRs, but their endogenous ligands have not yet been identified. In the next section characteristic features of the histatin-mediated cell activation will be discussed in relation to current concepts of GPCR-ligand interactions.

**Histatin as a ligand for a GPCR**

How binding of a signalling molecule may induce a cellular process is depicted in figure 1 of **Chapter 1**. Very briefly: when an activating ligand (agonist) binds to its receptor, it creates a conformational change of the receptor. For GPCRs, this results in the activation of G proteins, which is the first step in a cascade of signalling events that ultimately leads to a cellular activity, such as movement or cell division. After activation, the ligand-receptor complex is endocytosed, and targeted for lysosomal degradation or, after detachment of the ligand, the receptor is recycled to the membrane again (Figure 1, **Chapter 1**). It must be borne in mind that this scheme is an overly simplistic description. The interaction between a GPCR and its ligand is notoriously complex, and it is virtually impossible to formulate universal rules, due to the high diversity in both the receptors and the ligands. Variations on the basal theme have been demonstrated in virtually each stage of the process, from binding, to activation, to internalization. In particular complicating is that many GPCRs behave promiscuously, *i.e.* one and the same receptor can bind an extremely diverse set of ligands, initiate different cell-signalling pathways, and enter different endocytotic pathways.

**Mechanistics aspects of histatin-GPCR interaction.**

As explained above, the precise fit between receptor and ligand is the first crucial step in the GPCR signalling cascade. Ligands such as histatins, however, having in solution a random structure in general will fail to correctly fit into the binding site of its putative GPCR, and therefore will have a relative low affinity. This assumption is illustrated by the fact that the histatin concentration required for cell-activation, but also binding lies in the μM range (**Chapter 7**), which is well within the physiological concentration range occurring in saliva. On the other hand, polypeptides with a defined three-dimensional structure, such as EGF and SDF, have much higher affinities for their respective receptors and concomitant molar activities in the nM range. Cyclization of Hst1, which constrains its structural freedom, enhances its affinity and activity 1000-fold (**Chapters 4 and 7**). This indicates that the cyclic peptide has an increased propensity to adopt a bioactive conformation. Interestingly, a shift to a more ordered structure confirming such an increased propensity was not observed in the NMR structure analysis of cyclic Hst1 in solution (unpublished data). This obviously has implications for the molecular mechanism underlying the activation of the receptor by histatins. For this two extreme models can be envisioned:

(i) Conformational selection.

In this model the unbound (inactive) receptor makes rare excursions to the active form, to which the ligand (histatin) then binds, resulting in the activation of the signaling cascade, since that active form of the receptor sustains. Histatins have in solution a random structure, and therefore only a small fraction of the molecules will be present in a proper, bioactive conformation. Therefore, formation of a receptor-histatin complex, and subsequent activation, can only occur when both histatin and the receptor are in their active conformation. This model predicts that the enhanced activity of cyclic Hst1 is accompanied by a shift towards a more constrained ordered structure of the molecule in solution. However, high resolution NMR did not reveal differences in structure between linear and cyclic Hst1. Both peptides were in solution present as random-structured peptides with a large conformational flexibility.

(ii) Induced fit.

In this scenario, the ligand initially binds loosely to the receptor in its inactive form and subsequently induces the change to the active form, leading to the formation of the ligand-receptor complex. This model

explains the apparent discrepancy between the activity and the structural properties of cyclic Hst1; initially cyclic Hst1 (similar to linear Hst1) binds loosely and non-selectively to the inactive conformation of the receptor. In the binding site then apparently conditions are present which favour the transition to the bioactive conformation of the peptide. Synchronously, a transition of the receptor to its active conformation takes place. Peptides such as Hst5, which are virtually inactive but yet are internalized by the cell, can only perform the first step (loose binding to the inactive receptor), but cannot adopt the right conformation required to trigger a conformational change in the receptor to its active form. This initial binding is nevertheless sufficient for endocytosis, ostensibly similar to antagonist activities described by others (36). Figure 1 shows the two models in the different cases.

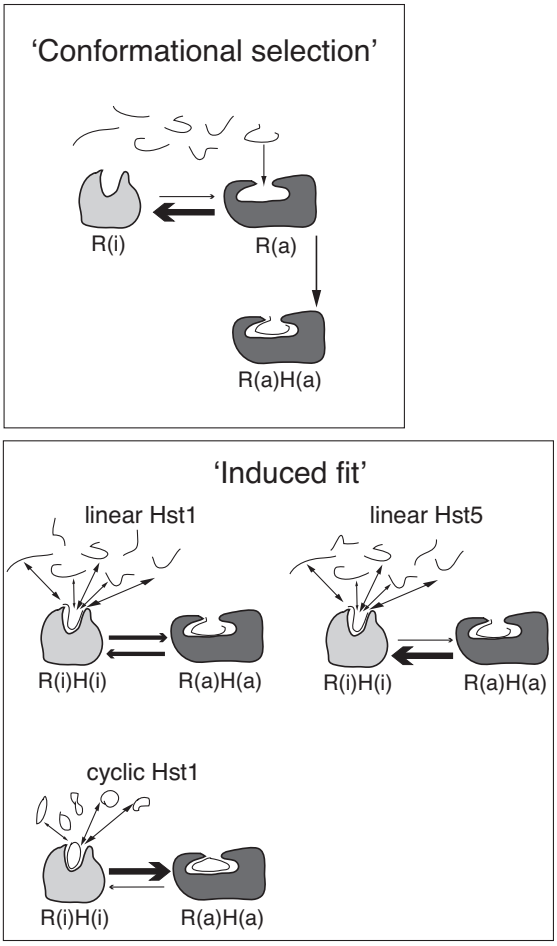


**Figure 1.** Activation models of receptor, comparison between 'conformational selection' and 'induced fit'

*Conformational selection*

In the absence of a ligand, the receptor in its inactive (R(i)) rarely converts into the active form (R(a)). The active conformation of the ligand (H(a)) binds to the active form of the receptor. In this way the active conformation of the receptor (R(a)) is stabilized, resulting in proper signalling.

*Induced fit*

The inactive receptor (R(i)) contains a binding site to which the ligand in its inactive conformation (H(i)) loosely (reversibly) binds. After binding of the ligand the receptor adopts its active conformation upon induction of the ligand. Illustrated in the figure are the moderate activity of linear Hst1, the highly active situation for cyclic Hst1, and the hardly active situation for Hst5.

## Comparative peptides for wound healing properties

Already in **Chapter 1** other antimicrobial peptides, such as LL-37 and defensins, are mentioned that also may influence wound healing. In **Chapter 6** we compare Hst2 and LL-37 in different aspects of wound healing and immune-modulation. The research on LL-37 in the context of wound healing and immune modulation is very extensive and complex. This complexity is largely because LL-37 acts on many different cells and commonly has a different effect depending on its concentration. For example, around 1 μM LL-37 enhances primary fibroblast proliferation, whereas at 10 μM LL-37 is very cytotoxic (**Chapter 6**). There seems to be a distinction between the effect of LL-37 on epithelial cells and fibroblasts, and its effect on cells derived from the bone marrow such as monocytes, neutrophils, dendritic cells, etc. Tomasinsig *et al.* (37) clearly shows that the activation of fibroblasts is not dependent on the direct interaction with a receptor. This is in accordance with other studies, with which together it has been shown that the D-enantiomer of LL-37 is equally active on the migration, proliferation, Il-8 response of fibroblasts and epithelial cells (37-39). The activity of LL-37 on these cells generally have a narrow optimum, in which slightly lower concentrations than this optimum do not activate, and at somewhat higher concentrations LL-37 is cytotoxic (**Chapter 6**, (37)). Nevertheless, the treatment of cells within the narrow optimum leads to transactivation of receptors such as $P2X_7$ and EGFR (37, 40, 41). Based on these findings, in our opinion, slight membrane disturbance activating epithelial and fibroblast cell types is the most plausible working mechanism. As is mentioned above, a different picture seems to emerge when cells that were originally derived from bone marrow are treated with LL-37. Interestingly, generally higher concentrations are required before LL-37 becomes cytotoxic when compared to epithelial cells or fibroblasts. LL-37 is starting to get toxic for leukocytes and lymophocytes from ~ 10 μM (42, 43), whereas it is toxic to fibroblasts from ~ 1 μM (**Chapter 6**, (44)). This difference in cytotoxic concentration has never been untangled, but for neutrophils it is thinkable that their extensive proteases repertoire could protect them against LL-37. Several cellular receptors have been proposed to act upon binding to LL-37, FPRL1 for neutrophils, monocytes and T cells (45), CXCR2 for neutrophils (46), and GAPDH as an intracellular receptor in monocytes (47). Unfortunately, no research group has published results with the D-LL-37 similar to the studies described above with epithelial cells and fibroblasts. However, Chen *et al.* (48) do show that the enhanced migration of monocytes by Frog-derived antimicrobial temporin A is stereo-specific and acts via the FPRL1 receptor, very similar thus to LL-37. Another indictation that LL-37 may activate these cells by directly activating the receptor

is that the narrow optimum that is seen with the activation of epithelial cells is less pronounced in bone-marrow derived cells (45, 48, 49). All in all, the function of LL-37 is not yet established. We think the *Cramp* knock-out mice model in which bacterial clearance was reduced (50), and, upon dysregulation by a serine protease, the association with the inflammatory skin disease rosacea (51) gives a good insight in the actual functional role of LL-37. It is important for the protection against infection, and its proper regulation is essential for proper inflammation responses.

In contrast to LL-37, histatins are continuously secreted by the salivary glands, and their abundance seemingly is not regulated upon a change of environment such as infection, or wounding for that matter. Rather, saliva is a versatile fluid that carries out a multitude of functions, often simultaneously (**Chapter 1**). And, saliva secretes a whole spectrum of proteins, each with its own function. The continuous presence of histatin thus can be seen as some sort of prophylactic, in contrast to the acute necessity for an antimicrobial agent and inducer of inflammation, by LL-37. Within this context, histatins probably continuously activate the epithelium, and this makes that the epithelium in the oral cavity responses very fast upon wounding. This may be one of the causes for the superior wound healing of the oral cavity compared to the skin.

## Clinical application of histatin in wound healing

We show in **Chapter 4** that histatin also enhances wound healing in a tissue-engineered skin wound model. This initial finding urges for many follow-up studies for eventual therapeutic use. Although from **Chapter 6** it can be deducted that histatin might not have adverse effects on scar tissue formation or inflammation, one needs to be quite careful implementing a research finding for clinical use. Wound healing is a very complex process that needs to be tightly regulated in order to function properly. With such a complex process it is not surprising that only a few factors that enhance healing via the direct activation of cells (and not a scaffold of some type) have made it to clinical trials. Currently, some growth factors (GM-CSF, bFGF, and TGF-$\beta_3$) are in clinical trials (52, 53). And the only FDA approved growth factor for clinical use is PDGF-BB, which is used in diabetic and pressure ulcers (52). The urgency for new factors is high, hard-to-heal chronic wounds represent a major burden in our society and significantly impair the quality of life of millions of people. The incidence of chronic wounds is expected to increase dramatically in the future due to ageing of the population and the increasing incidence of health-related disorders, such as diabetes, associated with

development of chronic wounds. The following considerations greatly contribute to the attractiveness of histatins as attractive lead compounds for the development of wound-healing enhancing therapeutics:

- Histatins are endogenous peptides, which make the occurrence of immunogenic side effects very unlikely.
- Histatins, thus far, are specifically acting on epithelial cells and fibroblasts, and not on inflammatory cells. The fact that they do not induce cell-proliferation limits the risk of uncontrolled cell division.
- Histatins are simple peptides, readily accessible to organic synthesis. In theory, this opens the door to fine-tuning of the cellular response by using synthetic variants.
- Histatins are interesting from a cost-effectiveness point of view. For bulk production of small peptides, organic synthesis is cheaper than recombinant production. Synthetic peptides are also easier to purify, without the risk of contamination with immunogenic microbial cell components. Truncation experiments revealed that large parts of the molecule can be missed without affecting the cell-stimulating activity (**Chapter 4**).
- Cyclization greatly enhances the cell-stimulating activity of histatins. In this respect, the novel method to produce semi-synthetic cyclic peptides by using recombinant *S. aureus* sortase A (**Chapter 7**) is very interesting. This method allows a very efficient, simple, and cheap production of biologically active peptides that are barely, if not at all, accessible to organic synthesis.

## Future prospects

The future is wide open for follow-up research on histatins with many interesting research questions available for in-depth and extensive studying.

First and most of all, the identification of the cellular receptor. By upscaling of the procedure described in **Chapter 7** (Figure 4), it will be possible to obtain sufficient quantities of the putative receptor of histatin to enable identification by mass-spectrometric techniques. A valuable tool for this is the sortase-based procedure to prepare cyclic histatin (**Chapter 7**), which makes it possible to produce the large quantities of cyclic histatin that are needed for immuno-affinity purification procedures. Alternatively, using histatin pepitdes equipped with (photoreactive) crosslinkers, specific binding partners of histatin can be crosslinked,

isolated, and identified.

Second, the role of histidine residues in histatin may be studied. Histatins are unusually rich in histidine, but the physiological meaning in the context of its role as cell-signalling molecule is unknown. Domain mapping of histatin (**Chapter 4**) reveals a minimal active domain containing merely one histidine residue. This suggests that the majority of histidine residues in histatin are not critical for cell-activating properties, making it tempting to speculate on another, for example regulatory, role. The imidazole side-group of histidine residues in histatins have pKa values ranging between 6.4 and 7.0 (54). Therefore, relatively small variations in the physiological pH-range will have profound effects on the charge, and possibly on the conformational and functional properties of histatins. pH in wounds may vary between < 6.0 and > 7.0, depending on the time course and wound stage (55). Thus far wound healing of histatin has been studied at neutral or slightly alkaline pH conditions. It would thus be interesting to examine the activity under pH conditions that occur in wounds during the different stages of the natural wound-healing process. Furthermore, it would be interesting to examine if compounds such as metal ions and tannins, which bind to histatin and putatively modify their structure, have an effect on their cell-activating properties.

Third, the effects of histatin on other clinically relevant processes may be studied. For application purposes, it is important to further explore the effect of histatins on all aspects of wound healing, such as inflammation and scar tissue formation. We briefly examined this in **Chapters 3 and 5**, and these preliminary results may be the starting point for studies using more complex wound-healing models. These follow-up studies are specifically interesting for pharmaceutical development, as histatins have several advantages over other compounds that are currently investigated for therapeutic utilization in wound healing, such as simple and cost-effective production and easily accesible for fine-tuning (see also the section 'clinical applications of histatins in wound healing' above).

Fourth, the interaction with the putative receptor of different (cyclic) histatin species could be studied. With our proposed receptor-activation model in mind (Fig. 1), and the clear distinction between endocytosis and binding and activation properties (**Chapters 6 and 7**), it would be interesting to pinpoint specific residues, or conformations that activate. Here again, the use of sortase to cyclisize peptides is a powerful tool that amplifies the possibilities, enabling NMR studies on the different peptides, because sufficient quantities can easily be prepared.

Fifth, are the signaling pathways that are utilized by histatins. In **Chapter 2** we describe that a Mek1/2 inhibitor specifically inhibits histatin-mediated cell

migration, and in **Chapter 4** we show that the activation is *Pertussis* toxin-sensitive. Combining these data suggest the activation of Erk1/2 by a GPCR, but this is most likely just a small part of the signaling routes utilized by histatin that eventually leads to cell migration. It thus would be interesting to study what exact signaling pathways are involved that ultimately lead to the induction of cell migration and cell spreading.

## References


1. Padovan, L., Segat, L., Pontillo, A., Antcheva, N., Tossi, A., and Crovella, S. (2010) Histatins In Non-Human Primates: Gene Variations and Functional Effects. *Protein Pept Lett*
2. Castagnola, M., Inzitari, R., Rossetti, D. V., Olmi, C., Cabras, T., Piras, V., Nicolussi, P., Sanna, M. T., Pellegrini, M., Giardina, B., and Messana, I. (2004) A cascade of 24 histatins (histatin 3 fragments) in human saliva. Suggestions for a pre-secretory sequential cleavage pathway. *J Biol Chem* 279, 41436-41443
3. Oppenheim, F. G., Xu, T., McMillian, F. M., Levitz, S. M., Diamond, R. D., Offner, G. D., and Troxler, R. F. (1988) Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on Candida albicans. *J Biol Chem* 263, 7472-7477
4. Baum, B. J., Bird, J. L., Millar, D. B., and Longton, R. W. (1976) Studies on histidine-rich polypeptides from human parotid saliva. *Arch Biochem Biophys* 177, 427-436
5. Siqueira, W. L., Margolis, H. C., Helmerhorst, E. J., Mendes, F. M., and Oppenheim, F. G. (2010) Evidence of intact histatins in the in vivo acquired enamel pellicle. *J Dent Res* 89, 626-630
6. Richardson, C. F., Johnsson, M., Raj, P. A., Levine, M. J., and Nancollas, G. H. (1993) The influence of histatin-5 fragments on the mineralization of hydroxyapatite. *Arch Oral Biol* 38, 997-1002
7. Oppenheim, F. G., Yang, Y. C., Diamond, R. D., Hyslop, D., Offner, G. D., and Troxler, R. F. (1986) The primary structure and functional characterization of the neutral histidine-rich polypeptide from human parotid secretion. *J Biol Chem* 261, 1177-1182
8. Yan, Q., and Bennick, A. (1995) Identification of histatins as tannin-binding proteins in human saliva. *Biochem J* 311 (Pt 1), 341-347
9. Naurato, N., Wong, P., Lu, Y., Wroblewski, K., and Bennick, A. (1999) Interaction of tannin with human salivary histatins. *J Agric Food Chem* 47, 2229-2234
10. Imatani, T., Kato, T., Minaguchi, K., and Okuda, K. (2000) Histatin 5 inhibits inflammatory cytokine induction from human gingival fibroblasts by Porphyromonas gingivalis. *Oral Microbiol Immunol* 15, 378-382
11. Imatani, T., Kato, T., Okuda, K., and Yamashita, Y. (2004) Histatin 5 inhibits apoptosis in human gingival fibroblasts induced by porphyromonas

gingivalis cell-surface polysaccharide. *Eur J Med Res* 9, 528-532

12. O'Brien-Simpson, N. M., Dashper, S. G., and Reynolds, E. C. (1998) Histatin 5 is a substrate and not an inhibitor of the Arg- and Lys-specific proteinases of Porphyromonas gingivalis. *Biochem Biophys Res Commun* 250, 474-478
13. Nishikata, M., Kanehira, T., Oh, H., Tani, H., Tazaki, M., and Kuboki, Y. (1991) Salivary histatin as an inhibitor of a protease produced by the oral bacterium Bacteroides gingivalis. *Biochem Biophys Res Commun* 174, 625-630
14. Gusman, H., Grogan, J., Kagan, H. M., Troxler, R. F., and Oppenheim, F. G. (2001) Salivary histatin 5 is a potent competitive inhibitor of the cysteine proteinase clostripain. *FEBS Lett* 489, 97-100
15. Gusman, H., Lendenmann, U., Grogan, J., Troxler, R. F., and Oppenheim, F. G. (2001) Is salivary histatin 5 a metallopeptide? *Biochim Biophys Acta* 1545, 86-95
16. Gusman, H., Travis, J., Helmerhorst, E. J., Potempa, J., Troxler, R. F., and Oppenheim, F. G. (2001) Salivary histatin 5 is an inhibitor of both host and bacterial enzymes implicated in periodontal disease. *Infect Immun* 69, 1402-1408
17. Mochon, A. B., and Liu, H. (2008) The antimicrobial peptide histatin-5 causes a spatially restricted disruption on the Candida albicans surface, allowing rapid entry of the peptide into the cytoplasm. *PLoS Pathog* 4, e1000190
18. Pollock, J. J., Denepitiya, L., MacKay, B. J., and Iacono, V. J. (1984) Fungistatic and fungicidal activity of human parotid salivary histidine-rich polypeptides on Candida albicans. *Infect Immun* 44, 702-707
19. MacKay, B. J., Pollock, J. J., Iacono, V. J., and Baum, B. J. (1984) Isolation of milligram quantities of a group of histidine-rich polypeptides from human parotid saliva. *Infect Immun* 44, 688-694
20. MacKay, B. J., Denepitiya, L., Iacono, V. J., Krost, S. B., and Pollock, J. J. (1984) Growth-inhibitory and bactericidal effects of human parotid salivary histidine-rich polypeptides on Streptococcus mutans. *Infect Immun* 44, 695-701
21. Xu, Y., Ambudkar, I., Yamagishi, H., Swaim, W., Walsh, T. J., and O'Connell, B. C. (1999) Histatin 3-mediated killing of Candida albicans: effect of extracellular salt concentration on binding and internalization. *Antimicrob Agents Chemother* 43, 2256-2262
22. Yin, A., Margolis, H. C., Grogan, J., Yao, Y., Troxler, R. F., and Oppenheim,

F. G. (2003) Physical parameters of hydroxyapatite adsorption and effect on candidacidal activity of histatins. *Arch Oral Biol* 48, 361-368

23. Johnsson, M., Levine, M. J., and Nancollas, G. H. (1993) Hydroxyapatite binding domains in salivary proteins. *Crit Rev Oral Biol Med* 4, 371-378
24. Melino, S., Rufini, S., Sette, M., Morero, R., Grottesi, A., Paci, M., and Petruzzelli, R. (1999) Zn(2+) ions selectively induce antimicrobial salivary peptide histatin-5 to fuse negatively charged vesicles. Identification and characterization of a zinc-binding motif present in the functional domain. *Biochemistry* 38, 9626-9633
25. Melino, S., Gallo, M., Trotta, E., Mondello, F., Paci, M., and Petruzzelli, R. (2006) Metal-binding and nuclease activity of an antimicrobial peptide analogue of the salivary histatin 5. *Biochemistry* 45, 15373-15383
26. Grogan, J., McKnight, C. J., Troxler, R. F., and Oppenheim, F. G. (2001) Zinc and copper bind to unique sites of histatin 5. *FEBS Lett* 491, 76-80
27. Flora, B., Gusman, H., Helmerhorst, E. J., Troxler, R. F., and Oppenheim, F. G. (2001) A new method for the isolation of histatins 1, 3, and 5 from parotid secretion using zinc precipitation. *Protein Expr Purif* 23, 198-206
28. Veerman, E. C., Valentijn-Benz, M., Nazmi, K., Ruissen, A. L., Walgreen-Weterings, E., van Marle, J., Doust, A. B., van't Hof, W., Bolscher, J. G., and Nieuw Amerongen, A. V. (2007) Energy depletion protects Candida albicans against antimicrobial peptides by rigidifying its cell membrane. *J Biol Chem* 282, 18831-18841
29. Xu, T., Levitz, S. M., Diamond, R. D., and Oppenheim, F. G. (1991) Anticandidal activity of major human salivary histatins. *Infect Immun* 59, 2549-2554
30. Molhoek, E. M., den Hertog, A. L., de Vries, A. M., Nazmi, K., Veerman, E. C., Hartgers, F. C., Yazdanbakhsh, M., Bikker, F. J., and van der Kleij, D. (2009) Structure-function relationship of the human antimicrobial peptide LL-37 and LL-37 fragments in the modulation of TLR responses. *Biol Chem* 390, 295-303
31. Mookherjee, N., Brown, K. L., Bowdish, D. M., Doria, S., Falsafi, R., Hokamp, K., Roche, F. M., Mu, R., Doho, G. H., Pistolic, J., Powers, J. P., Bryan, J., Brinkman, F. S., and Hancock, R. E. (2006) Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. *J Immunol* 176, 2455-2464
32. Scott, M. G., Davidson, D. J., Gold, M. R., Bowdish, D., and Hancock, R. E. (2002) The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *J Immunol* 169, 3883-3891

33. Brandenburg, K., Garidel, P., Fukuoka, S., Howe, J., Koch, M. H., Gutsmann, T., and Andra, J. (2010) Molecular basis for endotoxin neutralization by amphipathic peptides derived from the alpha-helical cationic core-region of NK-lysin. *Biophys Chem* 150, 80-87

34. Bowdish, D. M., Davidson, D. J., Scott, M. G., and Hancock, R. E. (2005) Immunomodulatory activities of small host defense peptides. *Antimicrob Agents Chemother* 49, 1727-1732

35. Basak, A., Ernst, B., Brewer, D., Seidah, N. G., Munzer, J. S., Lazure, C., and Lajoie, G. A. (1997) Histidine-rich human salivary peptides are inhibitors of proprotein convertases furin and PC7 but act as substrates for PC1. *J Pept Res* 49, 596-603

36. Simmons, G., Clapham, P. R., Picard, L., Offord, R. E., Rosenkilde, M. M., Schwartz, T. W., Buser, R., Wells, T. N., and Proudfoot, A. E. (1997) Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science* 276, 276-279

37. Tomasinsig, L., Pizzirani, C., Skerlavaj, B., Pellegatti, P., Gulinelli, S., Tossi, A., Di Virgilio, F., and Zanetti, M. (2008) The human cathelicidin LL-37 modulates the activities of the P2X7 receptor in a structure-dependent manner. *J Biol Chem* 283, 30471-30481

38. von Haussen, J., Koczulla, R., Shaykhiev, R., Herr, C., Pinkenburg, O., Reimer, D., Wiewrodt, R., Biesterfeld, S., Aigner, A., Czubayko, F., and Bals, R. (2008) The host defence peptide LL-37/hCAP-18 is a growth factor for lung cancer cells. *Lung Cancer* 59, 12-23

39. Braff, M. H., Hawkins, M. A., Di Nardo, A., Lopez-Garcia, B., Howell, M. D., Wong, C., Lin, K., Streib, J. E., Dorschner, R., Leung, D. Y., and Gallo, R. L. (2005) Structure-function relationships among human cathelicidin peptides: dissociation of antimicrobial properties from host immunostimulatory activities. *J Immunol* 174, 4271-4278

40. Tokumaru, S., Sayama, K., Shirakata, Y., Komatsuzawa, H., Ouhara, K., Hanakawa, Y., Yahata, Y., Dai, X., Tohyama, M., Nagai, H., Yang, L., Higashiyama, S., Yoshimura, A., Sugai, M., and Hashimoto, K. (2005) Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. *J Immunol* 175, 4662-4668

41. Tjabringa, G. S., Aarbiou, J., Ninaber, D. K., Drijfhout, J. W., Sorensen, O. E., Borregaard, N., Rabe, K. F., and Hiemstra, P. S. (2003) The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor. *J Immunol* 171,

6690-6696

42. Aarbiou, J., Tjabringa, G. S., Verhoosel, R. M., Ninaber, D. K., White, S. R., Peltenburg, L. T., Rabe, K. F., and Hiemstra, P. S. (2006) Mechanisms of cell death induced by the neutrophil antimicrobial peptides alpha-defensins and LL-37. *Inflamm Res* 55, 119-127
43. Johansson, J., Gudmundsson, G. H., Rottenberg, M. E., Berndt, K. D., and Agerberth, B. (1998) Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. *J Biol Chem* 273, 3718-3724
44. Shaykhiev, R., Beisswenger, C., Kandler, K., Senske, J., Puchner, A., Damm, T., Behr, J., and Bals, R. (2005) Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure. *Am J Physiol Lung Cell Mol Physiol* 289, L842-848
45. De, Y., Chen, Q., Schmidt, A. P., Anderson, G. M., Wang, J. M., Wooters, J., Oppenheim, J. J., and Chertov, O. (2000) LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* 192, 1069-1074
46. Zhang, Z., Cherryholmes, G., Chang, F., Rose, D. M., Schraufstatter, I., and Shively, J. E. (2009) Evidence that cathelicidin peptide LL-37 may act as a functional ligand for CXCR2 on human neutrophils. *Eur J Immunol* 39, 3181-3194
47. Mookherjee, N., Lippert, D. N., Hamill, P., Falsafi, R., Nijnik, A., Kindrachuk, J., Pistolic, J., Gardy, J., Miri, P., Naseer, M., Foster, L. J., and Hancock, R. E. (2009) Intracellular Receptor for Human Host Defense Peptide LL-37 in Monocytes. *J Immunol*
48. Chen, Q., Wade, D., Kurosaka, K., Wang, Z. Y., Oppenheim, J. J., and Yang, D. (2004) Temporin A and related frog antimicrobial peptides use formyl peptide receptor-like 1 as a receptor to chemoattract phagocytes. *J Immunol* 173, 2652-2659
49. Tjabringa, G. S., Ninaber, D. K., Drijfhout, J. W., Rabe, K. F., and Hiemstra, P. S. (2006) Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. *Int Arch Allergy Immunol* 140, 103-112
50. Nizet, V., Ohtake, T., Lauth, X., Trowbridge, J., Rudisill, J., Dorschner, R. A., Pestonjamasp, V., Piraino, J., Huttner, K., and Gallo, R. L. (2001) Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 414, 454-457
51. Yamasaki, K., Di Nardo, A., Bardan, A., Murakami, M., Ohtake, T., Coda,

A., Dorschner, R. A., Bonnart, C., Descargues, P., Hovnanian, A., Morhenn, V. B., and Gallo, R. L. (2007) Increased serine protease activity and cathelicidin promotes skin inflammation in rosacea. *Nat Med* 13, 975-980

52. Barrientos, S., Stojadinovic, O., Golinko, M. S., Brem, H., and Tomic-Canic, M. (2008) Growth factors and cytokines in wound healing. *Wound Repair Regen* 16, 585-601

53. Ferguson, M. W., Duncan, J., Bond, J., Bush, J., Durani, P., So, K., Taylor, L., Chantrey, J., Mason, T., James, G., Laverty, H., Occleston, N. L., Sattar, A., Ludlow, A., and O'Kane, S. (2009) Prophylactic administration of avotermin for improvement of skin scarring: three double-blind, placebo-controlled, phase I/II studies. *Lancet* 373, 1264-1274

54. Brewer, D., and Lajoie, G. (2002) Structure-based desing of potent histatin analogues. *Biochemistry* 41, 5526-5536

55. Schneider, L. A., Korber, A., Grabbe, S., and Dissemond, J. (2007 Influence of pH on wound-healing: a new perspective for wound-therapy? *Arch Dermatol Res* 298, 413-420